

DUAL TRANSCRIPTIONAL ACTIVITIES UNDERLIE OPPOSING EFFECTS OF
RETINOIC ACID IN MAMMARY CARCINOMA

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The vitamin A metabolite retinoic acid (RA) regulates potent anticarcinogenic activities and it is well established that these activities are mediated by retinoic acid receptors (RAR), ligand-inducible transcription factors that are members of the superfamily of nuclear hormone receptors. Transcriptional activation of RAR has been shown to trigger differentiation, cell cycle arrest, and apoptosis. However, in some cancers, RA facilitates rather than inhibits cell growth, but the basis for development of RA-resistance remains incompletely understood. Here we show that the pro-proliferative activities of RA are mediated through a different nuclear receptor, the peroxisome proliferator-activated receptor β/δ (PPAR β/δ). The data show that partitioning of RA between the two receptors is cell-specific: in RA-resistant cells, RA activates PPAR β/δ and functions as a survival factor, while in others, it operates through RAR and triggers apoptosis. We demonstrate that the partitioning RA between its two receptors is regulated by two intracellular lipid binding proteins, cellular retinoic acid binding protein II (CRABP-II) and fatty acid binding protein 5 (FABP5) which target RA to RAR and PPAR β/δ , respectively. The expression ratio of the two binding proteins in cells thus determines cellular responses to RA. The data demonstrate that both in cultured cells and in transgenic mice models of breast cancer, a high CRABP-II/FABP5 ratio targets RA to RAR, thus upregulating growth

inhibitory genes, including the cell cycle control protein BTG2, inducing the cleavage of the proapoptotic gene caspase 3, and suppressing carcinoma growth. Conversely, RA is targeted to PPAR β/δ in cells that express a low CRABP-II/FABP5 ratio, thereby enhancing transcription of pro-proliferative genes such as PDK-1, a key component of the Akt survival pathway, and acting as a pro-carcinogenic agent. Finally, we show that modulating the relative expression levels of the two binding proteins in cells and *in vivo*, results in profound changes in biological responses to RA, thus committing the cell to either life or death.

BIOGRAPHICAL SKETCH

Thaddeus T. Schug was born in Racine Wisconsin, the son of Fredrick and Tanya Schug. Thad attended the State University of New York at Potsdam where he received a B.A. degree with honors (cum laude) in Biology and Chemistry in 1989. Thad receives a Master of Arts in Teaching from Cornell University in 1993 and worked as a High School science teacher for the next decade. Thad returned to Cornell for his doctoral studies in 2004, joining the laboratory of Dr. Noa Noy.

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CHAPTER ONE

INTRODUCTION

1.1 Retinoid Transport and Metabolism:

Retinoic acid (RA), a metabolite of vitamin A, plays critical roles in embryonic development, and in growth and differentiation in adult mammals. Vitamin A is obtained through the diet as plant-derived provitamin A carotenoids, or as preformed retinyl esters from animal sources (Gamble, Shang et al. 1999; Vogel, Gamble et al. 1999). After absorption by mucosal cells in the intestine, carotenoids are converted to retinal by carotene cleavage enzyme. Retinal is then converted to retinol by retinal reductase. Dietary retinyl esters are hydrolyzed to yield free retinol and a free fatty acid through the action of retinyl ester hydrolases. In the enterocyte, retinol, originating from either source, is esterified to retinyl esters by lecithin: retinol acyltransferase (LRAT), packaged into chylomicrons and secreted into the lymphatic system. Retinoids are mainly stored in stellate cells (Ito cells) in the liver as retinyl esters in lipid droplets (Gamble, Shang et al. 1999; Vogel, Gamble et al. 1999). Upon depletion of serum vitamin A, retinol is mobilized and secreted to serum from these hepatic stores. It is well established that, except for the function of vitamin A in vision, which is dependent upon the 11-cis-retinaldehyde metabolite, most other biological roles of the vitamin are exerted by retinoic acids, which are synthesized from retinol through the action of two classes of enzymes: retinol dehydrogenases, which oxidize retinol to retinaldehyde, and retinal dehydrogenases which subsequently catalyze the oxidation of retinaldehyde to RA.

Due to their hydrophobic nature, retinoids are poorly soluble in aqueous environments and their solubilization and transport through aqueous phases is accomplished by their association with soluble proteins. Several proteins function to transport hydrophobic molecules in extracellular spaces, including serum albumin and retinol binding protein (RBP), which transports retinol in the blood. In the cell, the active metabolite of retinol, RA, is bound by CRABP-I, which is thought to facilitate RA catabolism, and CRABP-II, which, in the presence of ligand, has been shown to translocate to the nucleus and deliver RA to the ligand-activated transcription factors termed the retinoic acid receptors (RAR) (Dong, Ruuska et al. 1999; Budhu and Noy 2002; Sessler and Noy 2005).

In addition to CRABPs, the family of intracellular lipid-binding proteins (iLBP) also contains 9 known isotypes of proteins referred to as fatty acid binding proteins (FABP) which associate with a wide spectrum of long chain fatty acids and some of their metabolites. The exact functions and the nature of the physiologically meaningful ligands for these proteins are incompletely understood but it has been reported that, similarly to CRABP-II, some FABPs cooperate with specific ligand-activated transcription factors. Of special interest in the context of this thesis, it has been demonstrated that transcriptional activation by PPAR β/δ is supported by FABP 5 (FABP5, K-FABP, eFABP, mal1). Like other FABPs, FABP5 can bind various hydrophobic compounds, but it moves to the nucleus only upon association with ligands that activate PPAR β/δ . It thus shuttles cognate ligands from the cytosol to PPAR β/δ in the nucleus and enhances the receptor's activity (Tan, Shaw et al. 2002).

1.2 Nuclear Hormone Receptors:

The biological roles of the two active RA isomers, all-*trans*-RA and its 9-*cis* isomer, including regulation of cellular proliferation, differentiation and embryonic development are mediated through binding of two members of the family of nuclear hormone receptors, the retinoid X receptors (RXR) and RAR (Chambon 1996; Pfahl and Piedrafita 2003). Nuclear hormone receptors are ligand-activated transcription factors that bind to specific DNA sequences in the promoter region of inducible genes and act to modify gene transcription (Mangelsdorf, Thummel et al. 1995). The nuclear receptor family has been classified into 6 subclasses based on sequence homology and evolutionary considerations (Gronemeyer, Gustafsson et al. 2004). Subfamily 3 contains steroid receptors, such as estrogen receptor (ER), androgen receptor (AR), and glucocorticoid receptor (GR). Steroid receptors usually act as homodimers and interact with inverted DNA consensus sequences. Subfamilies 1 and 2 contain various receptors including the retinoid receptors RAR and RXR, vitamin D receptor (VDR), and peroxisome-proliferator-activated receptor (PPAR) (Gronemeyer, Gustafsson et al. 2004). The non-steroid receptors usually form heterodimers with a common binding partner, namely RXR. These heterodimers bind to DNA recognition sequences organized as direct repeats. Notably, some nuclear receptors termed orphan receptors to denote that the ligands that activate them (if such exist) are unknown. Work proposed here focuses on two nuclear receptors - RAR and PPAR β/δ .

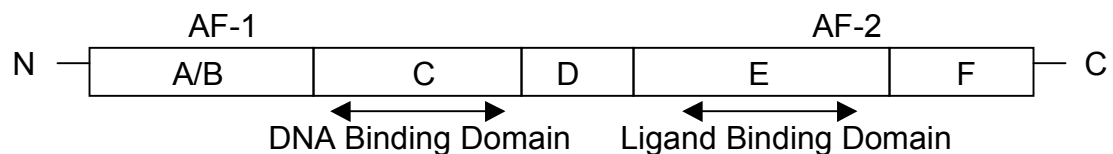


Figure 1.1 Illustration of functional domains of nuclear receptors

Nuclear hormone receptors share a common structural organization composed of modular domains A-F shown in figure 1. The amino terminal A/B region contains one of the receptors activation functions responsible for basal activation (i.e. ligand-independent activation) while a second ligand-dependent activation domain is located in the carboxy-terminal ligand-binding domain (LBD, region E)(Chambon 1996; van der Saag 1996). The LBD carries out multiple functions. It binds ligand, and contains a dimerization region as well as a region that mediates interactions with a variety of accessory proteins. Domain C contains two zinc finger motifs that allow for DNA binding by the receptor while the D domain is the hinge region conferring protein flexibility. The F domain is present in only some of the nuclear hormone receptors including RAR, however its function is unknown (Pfahl and Piedrafita 2003).

In the absence of ligand, nuclear receptors such as TR and RAR bind to their response elements and associate with corepressor complexes (*e.g.* nuclear receptor corepressor-NcoR and silencing mediator of retinoid and thyroid receptors-SMRT). Corepressor complexes keep the chromatin structure surrounding the promoter in a compact and inactive state (Blanco, Wang et al. 1995; Chen and Evans 1995). Upon ligand binding by the receptor, the corepressors dissociate and the receptor confers a transcriptionally active conformation. This active receptor signals transcription initiation by recruiting auxiliary proteins called coactivators. Initially, remodeling enzymes, such as the SWI/SNF complex, use the energy of ATP to reposition nucleosomes, exposing sites of transcription initiation (Horn and Peterson 2002; Lorch, Maier-Davis et al. 2006). Proteins that modify and open the chromatin template that, in turn, allows the basal transcription factors to bind the promoter. Proteins such as p300/CBP acetylate nucleosomal histones that loosen the chromatin template by catalyzing histone acetylation (Boube, Joulia et al. 2002). Other coactivators, such as

the Mediator (also known as thyroid hormone receptor associated proteins (TRAP) or vitamin D receptor interacting proteins (DRIP)), function to bridge receptors directly to the basal transcription machinery, stabilizing transcription initiation (Rachez, Gamble et al. 2000; Rachez and Freedman 2001). Mediator is a large, multi-subunit complex that contains members of the basal transcription machinery. Thus, ligand binding to the nuclear receptor is the critical step signaling initiation of transcription. Regulation of such a critical step will be an important aspect of cellular control.

Two class II nuclear hormone receptors, RAR and RXR, bind retinoic acids with distinct ligand-binding specificities. RAR is activated by both 9-*cis*-RA and all-*trans*-RA while RXR is activated by only 9-*cis*-RA (Giguere 1994). Both retinoid receptors exist as three isotypes (α , β , γ); each expressed from different genes and display different expression profiles, suggesting that they have distinct roles. Each isotype can be found as different isoforms as a result of differential promoter usage and alternative splicing (Leroy, Nakshatri et al. 1991).

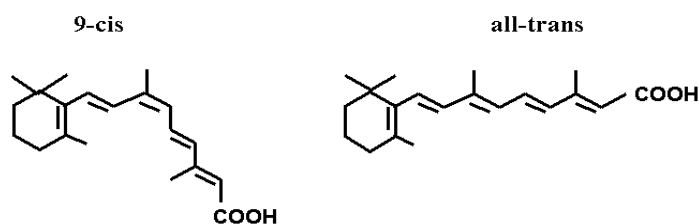


Figure 1.2 Structures of 9-*cis*-retinoic acid and all-*trans*-retinoic acid

RAR-RXR heterodimers bind to DNA response elements comprised of a direct repeat of the hexanucleotide half-site 5'-AGGTCA-3' separated by either two or five nucleotides (Umesono, Giguere et al. 1988). Target genes of retinoid receptors, such as *CYP26a*, control cellular processes involved in differentiation, apoptosis, and cell

cycle control. RXR functions as a common binding partner for all class II nuclear receptors and is considered the “master regulator” for non-steroid receptor signaling (Umesono, Giguere et al. 1988).

Most of the isoforms of both RARs and RXRs are expressed in breast cells. It has been reported that the retinoid receptor profiles in cells are altered during progression of various cancers (Gudas 1992). Most notably, the expression of RAR β 2 is markedly down-regulated in lung carcinoma, head and neck squamous cell carcinoma, and breast carcinoma. It was also reported that RA-dependent induction of RAR β correlates with the growth inhibitory effect of retinoids in several cancer cell lines, including some mammary carcinomas, and that RA-resistant breast cancer cells acquire RA sensitivity upon ectopic expression of RAR β (Verma, A. K., E. A. Conrad, et al. 1982). These observations suggest that the RAR β isoform is specifically involved in mediating the growth inhibitory effects of RA.

1.3 Peroxisome Proliferator Activated Receptors (PPARs):

Three different isotypes of PPAR are known to exist in mammals. These isotypes display different tissue distributions as well as cellular function. PPAR α is expressed in liver, kidney, muscle and heart, as well as in tissues with high rates of fatty acid catabolism. Consequently, activation of PPAR α lowers serum lipid levels, and synthetic ligands for this receptor are efficient therapeutic agents in treatment of hyperlipidemia (Forman, Tontonoz et al. 1995). PPAR γ is expressed predominantly in adipose tissue and macrophages, where it is involved in adipocyte differentiation, regulation of sugar and lipid homeostasis, and control of inflammatory responses (Devchand, Keller et al. 1996). PPAR β/δ is ubiquitously expressed with particularly

high level of expression found in brain, adipose tissue, skeletal muscle, and skin (Di-Poi, Tan et al. 2002). This receptor is involved in neuronal development, inflammation, skeletal muscle lipid oxidation, keratinocyte differentiation, epidermal barrier recovery, and lipid synthesis for keratinocyte proliferation (Icre et al., 2006; Michalik and Wahli; 2007). PPAR β/δ expression in skin is increased in hyperproliferative lesions, and in response to inflammatory cytokines during skin injury (Icre, Wahli et al. 2006; Michalik and Wahli 2007). Studies using transgenic mice have revealed evidence of PPAR involvement in numerous disease states (Desvergne and Wahli 1999).

Several biological lipids have been proposed to serve as endogenous biological ligands for PPARs. Recent studies have suggested that LTB₄ may have activity as an endogenous ligand for PPAR α . As PPAR α is involved in fatty acid oxidation, it has been suggested that LTB₄ acts in a negative feedback manner by inducing the transcription of genes involved its own metabolism (Devchand, Keller et al. 1996; Latruffe and Vamecq 1997). The cyclopentanone prostaglandin 15-deoxy PGJ₂ (15D-PGJ₂), which is the metabolite of the prostaglandin D₂, has been suggested to function as an endogenous ligand for PPAR (Forman, Tontonoz et al. 1995; Wayman, Ellis et al. 2002). However, the involvement of these and other potential ligands in the activities of PPARs *in vivo* has not been established, and these receptors remain classified as “orphan receptors”.

Our research project has centered primarily on PPAR β/δ . In contrast to PPAR α and PPAR γ , the physiological functions of PPAR β/δ are poorly understood. This may in part be due to the ubiquitous expression of PPAR β/δ as well as the difficulty in identifying its physiological ligands. However, synthetic ligands such as L-165041 and GW0470 have been shown to directly activate PPAR β/δ and induce downstream gene activity (Berger, Bailey et al. 1996).

PPAR β/δ was recently identified as a target of the tumor suppressor APC in colorectal cancer cells. PPAR β/δ is highly expressed in cells with inactive APC expression. A PPAR β/δ null colorectal cell line showed a significant reduction in ability to form tumors in nude mice compared to PPAR β/δ heterozygous colorectal cancer cells (He, Barak et al. 2003). PPAR β/δ expression in skin is increased in hyperproliferative lesions, and in response to inflammatory cytokines during skin injury. Elevation of PPAR β/δ expression in keratinocytes during skin injury is accompanied by production of an (unknown) endogenous ligand(s), resulting in protection against apoptotic signals and in enhancement of skin repair (Michalik and Wahli 2007). These anti-apoptotic activities are mediated, at least in part, by the ability of PPAR β/δ to directly upregulate the expression PDK1, thereby activating the survival factor Akt1 and protecting keratinocytes from apoptosis induced by cytokines such as TNF α (Di-Poi et al., 2002; Icre et al, 2006; Tan et al., 2004, Burdick et al, 2006).

1.4 Intracellular Lipid Binding Proteins:

It has been reported that several proteins in the intracellular lipid binding protein (iLBPs) family are capable of modulating the activities of NHRs. This protein family contains more than 14 members (see Table 1), including the fatty acid binding proteins (FABP), the cellular retinoic acid binding proteins (CRABP), and cellular retinol binding proteins (CRBPs) (Banaszak, Winter et al. 1994). The iLBPs have similar genomic organizations, consisting of four exons with total gene lengths of 5kB (Bernlohr, Simpson et al. 1997). The iLBPs are characterized by their similarity in size—roughly 14-16 KD, their highly conserved three-dimensional structure, and by their ability to bind small hydrophobic molecules (see Table 1)(Coe and Bernlohr

1998; Noy 2000). All members of this family bind a single hydrophobic ligand, except for L-FABP, which can bind two oleate molecules (Thompson, Ory et al. 1999).

FABPs include binding proteins, which were named according to the tissue they were first isolated in, are expressed abundantly in in cells involved in lipid metabolism, constituting 1-6% of total cytoplasmic protein (Bernlohr, Simpson et al. 1997). FABPs are more promiscuous than CRABPs and they bind a variety of fatty acids and fatty acid derivatives (Sweitzer, Love et al. 2000; Tan, Shaw et al. 2002) displaying ligand-binding selectivities reminiscent of those of PPARs. Thus, similar to PPARs the biological meaningful ligands for FABPs are unknown.

Table 1 iLBP tissue expression and distribution.

Common Name	Other Names	Expression
CRABP-I		Ubiquitously expressed in the adult and embryo
CRABP-II		Skin, uterus, ovary, and choroids plexus, widely expressed in the embryo
CRBP-I		Ubiquitously expressed in the adult tissues with high levels in the ovary, pancreas, pituitary ad adrenal glands, fetal liver
CRBP-II		Adult intestine, fetal kidney
CRBP-III		Adult kidney, liver, spleen, lymph nodes, and appendix
FABP2	IFABP	Intestine
FABP4	AFABP AP2	Adipose tissue, macrophages

Table 1 (Continued)

FABP5	KLBP eFABP FABP5 Mal1	Endothelial cells, adipose tissue, lens, lung, brain, mammary glands, placenta, heart, skeletal muscle, intestine, testis, retina, and abnormal skin
FABP1	LFABP	Liver, intestine, kidney, and lungs
FABP3	M-FABP MDGI HFABP	Heart, mammary gland, and skeletal muscle
FABP7	BLBP MRG BFABP	Brain and olfactory bulb
FABP6	BABP ILBP Gastrotropin	Ileum
FABP9	TLBP	Testis
FABP8	MLBP PMP2 My-FABP	Schwann cells

Table 1 iLBP tissue expression and distribution.

CRABPs display distinct tissue distribution; CRABP-II, which is widely distributed in the embryo, is expressed in the adult mainly in keratinocytes, in sertoli cells in the testis, at the uterus at particular stages of the estrus cycle and during pregnancy, and in the choroid plexus (Ong, Newcomer et al. 1994; Zheng and Ong

1998). In contrast, CRABP-I is widely distributed in the adult (Ong, Newcomer et al. 1994). Both proteins bind specifically to all-*trans*-RA with K_d values of 0.06 nM and 0.13 nM for CRABP-I and CRABP-II, respectively (Dong, Ruuska et al. 1999). A sub family of the intracellular lipid binding proteins is the family of fatty acid binding proteins (FABPs) which were named according to the tissue they were first isolated in. Currently there are nine known members of the FABP family.

1.5 CRABP and FABP Function:

Traditionally, it has been thought that FABPs play a passive role in solubilizing their ligand (Ong, Newcomer et al. 1994). However, it is becoming increasingly clear that these binding proteins play more specific roles in modulating signaling by these ligands. The presence of CRABP-I has been shown to correlate with a decreased ability of F9 teratocarcinoma cells to differentiate in response to RA and with enhanced RA degradation (Gudas 1992). Therefore, it has been suggested that CRABP-I functions to activate RA degradation pathways or to sequester it from binding to nuclear receptors. A more specific role for this protein has yet to be discovered.

Current evidence suggests that CRABP-II functions by directly delivering RA to RAR in the nucleus, thereby facilitating the ligation of the receptor and augmenting its transcriptional activities (Dong, Ruuska et al. 1999; Budhu and Noy 2002; Sessler and Noy 2005). Our lab has shown the mechanism by which CRABP-II translocates from the cytosol to the nucleus upon binding of RA leading to a transcriptional enhancement of RAR. Our lab and others have also found that CRABP-II enhances the RA-induced transcriptional activity of RAR in cells. The ability of CRABP-II to

potentiate activity of RAR stems from its ability to channel RA to the receptor, which results in facilitation of the formation of the active form of RAR.

It has been demonstrated that over-expression of CRABP-II dramatically enhances the responsiveness of mammary carcinoma cells to RA-induced growth inhibition, while diminished expression of the protein results in RA resistance (Budhu and Noy 2002; Donato and Noy 2005). We have also shown that CRABP-II inhibits mammary tumor growth in two different mouse models of cancer that are known to be RA-resistant. Our observations, as well as others, indicate that the level of expression of CRABP-II in several breast cancer cell lines and mouse models correlates with the efficiency of RA-induced growth inhibition, suggesting that down-regulation of CRABP-II is associated with development of RA resistance (Budhu and Noy 2002; Manor, Shmidt et al. 2003).

The evidence suggesting that CRABP-II acts as a coregulator of RAR raises the possibility that other members of the iLBP family may cooperate with nuclear receptors. It was reported that some FABPs act in concert with PPARs and that this activity is highly selective for particular FABP-PPAR pairs: FABP3 activates PPAR α , FABP4 specifically enhances the activity of PPAR γ , while FABP5 activates PPAR β/δ (Tan, Shaw et al. 2002). It was shown that both the ligand-induced nuclear localization of the FABPs and their ability to enhance transcriptional activity are highly selective for the ligand of a particular PPAR isotype. This is so despite the apparent lack of selectivity in ligand binding by the FABPs. These observations suggest that FABPs employ different modes of binding toward different ligands (Tan, Shaw et al. 2002). Interestingly, it has been reported that RA binds to PPAR β/δ with nanomolar affinity, modulates the conformation of the receptor, promotes interaction with the coactivator SRC-1, and efficiently activates PPAR β/δ -mediated transcription (Shaw, Elholm et al. 2003). These observations suggest that RA may be functioning through two distinct

nuclear receptors, the classical RA receptor RAR and PPAR β/δ . The involvement of iLBPs in the transcriptional activities of nuclear receptors suggest further that regulation of the transcriptional activity of RA may be mediated by the iLBPs that specifically cooperate with RAR and PPAR β/δ , namely CRABP-II and FABP5.

1.6 Mouse Model:

To examine the roles of RA in mammary cancer development, we used the transgenic mouse model of breast cancer MMTV-*neu*. In these mice, *Neu/Erb-B2*, which encodes for a member of the growth factor tyrosine kinase receptor family, is specifically over-expressed in mammary tissue, resulting in spontaneous development of mammary tumors in 100% of female mice (Guy, Webster et al. 1992).

Amplification of this gene has been observed in a significant proportion of primary human breast cancers (King, Kraus et al. 1985; Yokota, Yamamoto et al. 1986), and a strong correlation has been established between the extent of overexpression and poor outcome in human patients (Slamon, Godolphin et al. 1989).

Notably, over-expression of *Neu* induces profound RA-resistance (Tari, Lim et al. 2002). The basis for this resistance is currently unknown. However, previous observations demonstrated that injection of an adenovirus harboring CRABP-II into tumors that arise from MMTV-*neu* mice results in a significant delay in tumor development (Manor, Shmidt et al. 2003). It was further shown that administration of RA in the presence of CRABP-II over-expression had no additional beneficial effects, and suggested that RA treatment in the absence of ectopic over-expression of CRABP-II may have facilitated tumor growth. Taken together, these observations raise the intriguing possibility that RA inhibits tumor growth in the presence of CRABP-II,

which delivers it to RAR, but facilitates tumor development in the absence of this binding protein. Interestingly, such opposing effects of RA on cell growth was also noted in other studies. While RA displays distinct anticarcinogenic activities when associated with RAR activation, it appears to promote rather than inhibit cell survival in some cell types and tissues (Verma, Conrad et al. 1982; Rodriguez-Tebar and Rohrer 1991; Henion and Weston 1994; Plum, Parada et al. 2001). For example, RA is critical for neural survival both in cultured cell models and *in vivo*. It was also shown that RA treatment can enhance keratinocyte proliferation (Verma, Conrad et al. 1982). The main goal of work described here was to delineate the molecular basis for these opposing activities of this hormone.

1.7 Research Goals:

The central hypothesis of this work is that RA activates two different nuclear receptors, RAR and PPAR β/δ , and that alternate activation of these receptor results in distinct cellular responses. We hypothesize further that the partitioning of RA between its two receptors is regulated by two iLBPs, CRABP-II which delivers it to RAR and FABP5 which shuttles the hormone to PPAR β/δ . To address these hypotheses, the work had two specific goals.

1. To investigate the roles of RA and its associated nuclear receptors and intracellular lipid binding proteins in regulation of cell growth.

While activation of RAR by RA usually results in inhibition of cell growth, it has been reported that, in some circumstances, RA may operate through an RAR-independent pathway to promote cell survival and enhance proliferation. Several cell

lines will be used to examine whether the RAR-independent, pro-proliferative activities of RA are mediated by PPAR β/δ . Additional experiments will be carried out to investigate the possibility that CRABP-II and FABP5 regulate the partitioning of the hormone between the two receptors, and thus biological responses to RA.

2. To investigate the involvement of RA and its associated nuclear receptors and intracellular lipid binding proteins in tumor development in mammary cancer mouse models.

To obtain insight into the involvement of the two RA receptors in tumor development in MMTV-*neu* mice, and to further examine the possibility that CRABP-II and FABP5 regulate biological responses to RA, MMTV-*neu* mammary carcinoma mouse models that express varying expression ratios of the FABP5/CRABP-II will be generated. Tumors that develop in these models will be analyzed as to transcriptional activities of RAR and PPAR β/δ , and as to characteristics of tumor development and histology.

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CHAPTER TWO

OPPOSING EFFECTS OF RETINOIC ACID ON CELL GROWTH RESULT FROM ALTERNATE ACTIVATION OF TWO DIFFERENT NUCLEAR RECEPTORS*

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2.1 Summary

Transcriptional activation of the nuclear receptor RAR by retinoic acid (RA) often leads to inhibition of cell growth. However, in some tissues, RA promotes cell survival and hyperplasia, activities that are unlikely to be mediated by RAR. Here we show that, in addition to functioning through RAR, RA activates the ‘orphan’ nuclear receptor PPAR β/δ , which, in turn, induces the expression of pro-survival genes. Partitioning of RA between the two receptors is regulated by the intracellular lipid-binding proteins CRABP-II and FABP5. These proteins specifically deliver RA from the cytosol to nuclear RAR and PPAR β/δ , respectively, thereby selectively enhancing the transcriptional activity of their cognate receptors. Consequently, RA functions through RAR and is a pro-apoptotic agent in cells with high CRABP-II/FABP5 ratio, but it signals through PPAR β/δ and promotes survival in cells that highly express FABP5. Opposing effects of RA on cell growth thus emanate from alternate activation of two different nuclear receptors.

2.2 Introduction

The vitamin A metabolite retinoic acid (RA) regulates multiple biological processes and plays key roles in embryonic development and in tissue remodeling in the adult. It is well established that many of the activities of RA are mediated by retinoic acid receptors (RAR α , β , and γ), ligand-inducible transcription factors that are members of the superfamily of nuclear hormone receptors (Laudet and Gronemeyer, 2002). RARs associate with the retinoid X receptor (RXR) to form heterodimers that bind to regulatory regions of specific target genes and modulate transcriptional rates in response to their cognate ligands (Chambon, 1996; Mangelsdorf et al., 1994).

Transcriptional activation by RAR may trigger differentiation (Park et al., 1999; Rochette-Egly and Chambon, 2001), cell cycle arrest (Donato et al., 2007), and apoptosis (Altucci et al., 2001; Donato and Noy, 2005; Kitareewan et al., 2002), and thus often leads to inhibition of cell proliferation. Accordingly, RA displays distinct anticarcinogenic activities and is currently used in or is being tested as a therapeutic agent in several human cancers (Soprano et al., 2004).

However, in some tissues, RA appears to promote rather than inhibit cell survival. For example, RA is critical for neuronal survival (Henion and Weston, 1994; Jacobs et al., 2006; Plum et al., 2001; Rodriguez-Tebar and Rohrer, 1991), and it was reported that RA treatment can enhance skin tumor formation (Verma et al., 1982). RA is essential for normal keratinization in various epithelia (Wolbach and Howe, 1978), and topical administration of the hormone stimulates dermal repair and induces hyperproliferation of basal keratinocytes (Kang et al., 1995; Zouboulis, 2001). Interestingly, although RA plays critical roles in maintenance of skin integrity, mice lacking both $RAR\alpha$ and $RAR\gamma$ as well as $RAR\beta$ -null mice display normal keratinocyte proliferation (Chapellier et al., 2002). These observations indicate that RARs are dispensable for keratinocyte renewal, and suggest that some RA activities in the skin are mediated by an RAR-independent pathway.

We previously reported that RA binds with a high affinity to another nuclear receptor, namely $PPAR\beta/\delta$, a member of a sub-class of receptors which also includes $PPAR\alpha$ and $PPAR\gamma$, and that, like RAR, functions as a heterodimer with RXR (Laudet and Gronemeyer, 2002). Selective association of RA with $PPAR\beta/\delta$ was suggested by the observations that the K_d for RA-binding by this receptor is ~ 15 nM, about an order of magnitude stronger than that displayed by $PPAR\alpha$ and $PPAR\gamma$. Correspondingly, in the context of a reporter gene construct, RA was found to efficiently activate $PPAR\beta/\delta$ but not $PPAR\alpha$ or $PPAR\gamma$ (Shaw et al., 2003). Hence, an intriguing possibility is that, in some cells, RA may activate transcription

not only through RAR but also through PPAR β/δ . It is noteworthy in regard to this that, in keratinocytes, PPAR β/δ induces differentiation and, importantly, displays pronounced anti-apoptotic activities mediated in part by direct transcriptional targeting of the PDK-1/Akt survival pathway (Di-Poi et al., 2002; Tan et al., 2004). Consequently, PPAR β/δ is central to maintenance of skin permeability-barrier integrity, and to keratinocyte survival during inflammation and wound healing (Di-Poi et al., 2003; Icre et al., 2006).

Ligands that activate RAR and the various PPAR isotypes also bind in cells to intracellular lipid binding proteins (iLBPs), a family of small proteins that share a remarkably similar 3-dimensional structure (Gutierrez-Gonzalez et al., 2002; Kleywegt et al., 1994; Veerkamp and Maatman, 1995), but bind lipophilic molecules with distinct selectivities. Some members of this family, termed cellular retinoic acid binding proteins (CRABP-I and II), specifically associate with retinoic acid with subnanomolar affinities (Dong et al., 1999). Other iLBPs, known as fatty acid binding proteins (FABPs), display broad selectivities and bind a variety of fatty acids and some fatty acid derivatives. In fact, the spectrum of ligands that bind to FABPs is reminiscent of that of PPARs (Gutierrez-Gonzalez et al., 2002; Hanhoff et al., 2002; Norris and Spector, 2002; Widstrom et al., 2001). The shared ligand selectivities of some iLBPs and some nuclear receptors suggest that specific members of the two classes of proteins may cooperate in regulating the biological activities of their common ligands. Such a cooperation is also suggested by overlapping tissue expression profiles and by involvement in similar biological functions.

Recent studies indeed demonstrated that three iLBPs, CRABP-II, FABP5 (K-FABP, eFABP, mal1), and FABP4 (A-FABP, aP2), selectively cooperate with the nuclear receptors RAR α , PPAR β/δ and PPAR γ , respectively. Specifically, these studies established that, upon association with particular ligands, these binding proteins translocate from the cytosol to the nucleus, that they engage in direct protein-

protein interactions with their ‘cognate’ receptors, and that the resulting complex mediates ‘ligand-channeling’ from the binding protein to the receptor. Consequently, the binding proteins facilitate the ligation of the respective receptors and significantly augment their transcriptional activities (Budhu and Noy, 2002; Dong et al., 1999; Manor et al., 2003; Sessler and Noy, 2005; Tan et al., 2002). Interestingly, although FABP4 and FABP5 bind multiple ligands, only particular compounds trigger their nuclear translocation (Tan et al., 2002). FABP4 moves into the nucleus in response to ligands that activate PPAR γ but not upon treatment with PPAR β/δ ligands. In contrast, FABP5 mobilizes to the nucleus only in response to ligands that activate PPAR β/δ .

Hence, available information suggests the possibility that, while RA inhibits cell- growth by signalling through RAR, the ‘non-traditional’ pro-proliferative activities of this hormone may be mediated by PPAR β/δ . Work described in this manuscript was undertaken in order to examine this hypothesis, and to investigate the possibility that CRABP-II and FABP5 control the partitioning of RA between the two receptors.

2.3 Experimental Procedures

Reagents CRABP-II antibodies were provided by Pierre Chambon (IGMCB, Strasbourg, France). FABP5 antibodies were purchased from BioVender (Candler, NC). Antibodies against full-length and cleaved PARP, and total and phospho-Akt (Thr308) were from Cell Signaling Technology. Anti-mouse and anti-rabbit immunoglobulin antibodies conjugated to horseradish peroxidase were from Amersham (Arlington Heights, IL) and BioRad (Hercules, Ca), respectively. Anilidonaphtalene-8-sulphonic acid (ANS), RA, TNF α , and TRAIL were from Sigma Chemical Co. (St. Louis, MO). 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic Acid (TTNPB) and GW0742 were purchased from Biomol International (Plymouth Meeting, PA) and Toronto Research

Diagnostics Inc. (Toronto, ON), respectively..

Proteins. Histidine-tagged CRABP-II and GST-tagged FABP5 were expressed in the *E. coli* strain BL21. Bacteria were grown overnight at 25°C and protein expression was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) overnight. Bacteria were pelleted and lysed in lysis buffer (20 mM Tris, pH 8.0, 0.5 mM NaCl, 100 μ M phenylmethylsulfonyl fluoride) containing lysozyme and DNase I. Mixtures were incubated (30 min., 37°C), centrifuged, and proteins purified by affinity chromatography, and dialyzed against lysis buffer.

Cells. COS-7, HaCaT, NaF, and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal-treated newborn bovine calf serum (Cocalico Biologicals Inc., Reamstown, Pa.). COS-7 cells were transfected using Fugene (Roche Diagnostics Corporation). Other cell lines were transfected using Superfect (Qiagen).

Carcinogenesis studies in the MMTV-*neu* mice. A colony of the homozygous strain TgN(*MMTVneu*)202Mul mice was maintained on FVB/N background. All experiments were performed on virgin females. Tumor development was monitored by palpation three times a week, and tumor sizes were recorded without knowledge of the applied treatment. RA was administered by 90-day release 15 mg pellets (Innovative Research of America, Sarasota, Florida) implanted subcutaneously on age 140 days. Statistical analyses of tumor growth curves were performed on the natural log of tumor volumes in the two groups and compared with an unpaired t test using MINITAB software. P value for comparison between survival curves was calculated using the Kaplan-Meier method.

Transactivation assays. Cells were cultured in 24-well plates and co-transfected with (PPRE)₃-luciferase reporter vector (100 ng), a vector harboring the appropriate iLBP (in pCDNA 3.1, 200 ng), and pCH110, a β -galactosidase expression plasmid (50 ng). In some experiments, an expression vector encoding RAR or PPAR β/δ (in

pSG5, 50 ng) was cotransfected. Transfections were carried out using Fugene (Roche Diagnostics Corporation) according to the protocol of the manufacturer. Twenty-four hours following transfection, medium was replaced by DMEM, and ligands were added (RA – in ethanol, other ligands - in dimethylsulfoxide). Following 24 hr of treatment, cells were lysed and lysates assayed for luciferase activity (Luciferase assay system, Promega) which was corrected for β -galactosidase activity.

Experiments were carried out in triplicates.

Apoptosis was evaluated using the APOPercentage Apoptosis Assay kit (Biocolor Ltd. United Kingdom). 1×10^6 cells were suspended in 1 ml medium and dispensed into 96-well microplates. Cells were grown overnight, treated with appropriate ligand (2 hr.) and apoptosis induced with TNF α or TRAIL overnight. Medium was replaced with medium containing APOPercentage Dye Label. The APOP% Dye Release Reagent was added and a microplate colorimeter was used to measure cell-bound dye recovered in solution. Apoptotic index was measured at λ - 595 nm.

Binding assays were carried out by fluorescence titrations using a Fluorolog 2 DMIB spectrofluorometer (SPEX Instruments, Edison, N.J.). FABP5 was bacterially expressed and purified and the equilibrium dissociation constants (K_d) that characterize its interactions with RA and GW0742 were measured by fluorescence competition assays. The method entails two steps (Lin et al., 1999). In the first step, K_d for the association of the protein with the fluorescent fatty acid probe ANS was measured. Protein ($1 \mu\text{M}$) was titrated with ANS from a concentrated solution in ethanol. Ligand binding was monitored by following the increase in the fluorescence of the ligand upon binding to the protein (λ_{ex} -370 nm; λ_{em} -475 nm). Titration curves were analyzed (Norris and Li, 1998) to yield the number of binding sites and K_d . K_d s for binding of non-fluorescent ligands were then measured by monitoring their ability to compete with fluorescent probes for binding. The protein was pre-complexed with ANS at 1:1 molar ratio and titrated with RA or GW0742 whose

binding was reflected by a decrease in probe fluorescence. K_ds were extracted from the EC₅₀ of the competition curve and the measured K_d for the probe. Analyses were carried out using Origin 7.5 software (MicroCal Software Inc., Northampton, Mass.).

Quantitative real-time PCR (Q-PCR). RNA was extracted and cDNA generated using Gene Amp RNA PCR (Applied Biosystems, Foster City, CA). Q-PCR analyses for PDK-1 were conducted using TaqMan chemistry and Assays on Demand probes (Applied Biosystems, PDK1 - Hs00176884_m1, ADRP - Hs00765634_m1, FIAF - Hs00211522_m1), Cyp26a – Hs01075671_g1, BTG2 – Hs00198887_m1, cyclin D1 0 Hs00277039-m1. 18S ribosomal RNA (4319413E-0312010) was used as a loading control. Analyses were carried out using the relative standard curve method (Applied Biosystems Technical Bulletin no. 2).

Confocal fluorescence microscopy. COS-7 cells were plated in 35 mm glass bottom microwell dishes (Mattek) in DMEM containing 5% charcoal-treated FBS (75,000 cells per plate), grown for 12 hr. and transfected using Fugene (Roche) with an expression vector harboring GFP-tagged FABP5 (EGFP, 250 ng DNA per plate). Following a 48 hr incubation, medium was replaced with serum-free DMEM, and live cells were imaged using a Leica TCS SP2 confocal microscope equipped with a 40x oil immersion lens. After imaging, cells were treated with ligands (1 μ M), incubated at 37°C for 30 min and imaged again. An average of 40 cells were analyzed using ImageJ (National Institute of Health).

Chromatin immunoprecipitation assays. Nearly confluent HaCat cells were treated with vehicle or RA (1 μ M, 45 min.). Proteins were cross-linked to DNA (1% formaldehyde, 10 min.). Cells were washed with PBS, scraped, collected, lysed (1% SDS, 10 mM EDTA, 50 mM Tris, pH 7.9, 1 mM DTT, and protease inhibitors (Roche), and incubated on ice (45 min.). Samples were sonicated three times, and chromatin precleared with protein A beads (2 hr.). Antibodies against PPAR β/δ (H-

74, Santa Cruz) or pre-immune rabbit IgG were added and mixtures incubated overnight at 4°C. Protein A beads were added and mixed (2 hr., 4°C). Beads were washed twice with low-salt buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl), twice with high salt buffer (low salt buffer + 500 mM NaCl), and twice with Tris-EDTA buffer. Cross-link was then reversed (100 mM NaHCO₃, 1% SDS, overnight 65°C), proteins digested with proteinase K (1 hr.), and DNA purified (nucleotide extraction kit, Qiagen). The PPRE containing regions of *ADRP* (<http://www.nuclear-receptor.com/content/pdf/1478-1336-3-3.pdf>) and *FIAF* (<http://www.jbc.org/cgi/reprint/279/33/34411>) were amplified by PCR using the following primers. *ADRP*: 5'-CCTCTGCTTCACAGGCAAATA -3' (forward) and 5'-TGCATCAGAAGACTCTCGCCCTTT-3' (reverse); *FIAF*: 5'-AATCATGGAAGCCACACTGGTGGT -3' (forward) and 5'-CCCTACTTTCCTCCCATCCAGTAA-3' (reverse). Primers specific for a region 6-kb upstream of the GAPDH promoter, 5'-TCACGCCTGTAATCCCAGCACTTT -3' (forward) and, 5'-TGATTTCGGCTCACTACAACCTCC-3' (reverse), were used as a negative control.

2.4 Results

RA facilitates tumor growth in the MMTV-*neu* transgenic mammary cancer model. In keratinocytes, RA exerts pro-proliferative activities that appear to be mediated by a pathway other than activation of RAR (Chapellier et al., 2002). In search for an additional model that will allow for exploring the mechanisms that underlie 'non-traditional' activities of RA, we considered the transgenic mammary cancer mouse model TgN(*MMTVneu*)202Mul (Akagi et al., 1997; Guy et al., 1992).

The oncogenic hallmark of this model is a mammary-specific amplification of the growth factor receptor c-erb-B2/*neu*, which is often amplified in primary human

breast cancers (King et al., 1985). This model, in which 100% of female mice develop mammary adenocarcinomas (Muller et al., 1988), was chosen because it has been reported that over-expression of neu in mammary carcinoma cells leads to RA-resistance, suggesting down-regulation of RAR signalling (Tari et al., 2002). To examine the effects of RA on tumor development, the rate of tumor growth was

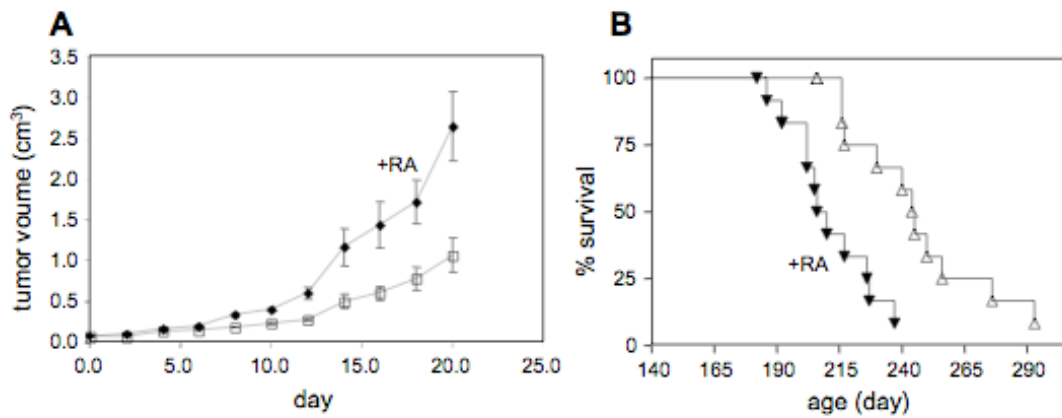


Figure 2.1. RA facilitates tumor growth in *MMTV-neu* mice. Tumor growth was monitored in untreated female *MMTV-neu* mice and in mice subjected to systemic RA as of age 140 d. **A.** Sizes of tumors were measured with calipers with a start point (day 0) of tumor volume = 0.065 cm³. Volumes of tumors in untreated (closed symbols) and RA-treated (open symbols) on day 20 were 1.09±0.22 cm³ vs. 1.87±0.25 cm³ (mean±SEM; n=12 in each group, P = 0.04). **B.** Mice were treated as described in **a.**, and survival, defined as the age in which tumor volume reached 0.524 cm³, was assessed. Median survivals were 240 and 205 days for untreated mice (open symbols) and RA-treated mice (closed symbols, n=12 in each group, P = 0.001).

studied in untreated mice and in mice systemically treated with RA as of age 140 days. Measurements were initiated when tumors reached a volume of 0.065 cm^3 . The data (Fig. 2.1A) showed that RA treatment dramatically facilitated tumors formation with mean tumor volume on day 20 of $1.09 \pm 0.22 \text{ cm}^3$ in untreated mice, and $1.87 \pm 0.25 \text{ cm}^3$ in the RA-treated group (mean \pm S.E.M., $n = 12$ in each group, $P = 0.04$). Assessment of mouse survival, defined as the time when tumor volume reached 0.524 cm^3 (Fig. 2.1B), indicated that, although all mice eventually developed tumors of that size, the median survival of RA-treated vs untreated mice was 205 vs 240 days ($P = 0.001$). Hence, RA exerts pro-proliferative activities in tumors that arise in MMTV neu mice.

Subsequent experiments thus utilized two cell lines in which RA displays ‘non-traditional’ activities, leading to cell survival and growth: HaCaT keratinocyte cells, and NaF cells, derived from tumors that arise in MMTV neu mice. For comparison, we employed MCF-7 mammary carcinoma cells, in which RA is known to function through RAR and to inhibit cell proliferation (Donato and Noy, 2005; Donato et al., 2007; Mangiarotti et al., 1998; Toma et al., 1998).

In HaCaT keratinocytes, RA activates PPAR β/δ in parallel to activation of RAR. The ability of RA to activate PPAR β/δ in the human keratinocyte cell line HaCaT was examined. Transcriptional activation assays conducted using a luciferase reporter driven by a consensus PPAR response element (PPRE) showed that the synthetic PPAR β/δ -selective ligand GW0742, but not the RAR-selective ligand 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), induced transcription of the reporter (Fig. 2.2A). These observations attest to the expression and functionality of PPAR β/δ in these cells and demonstrate specificity of reporter response.

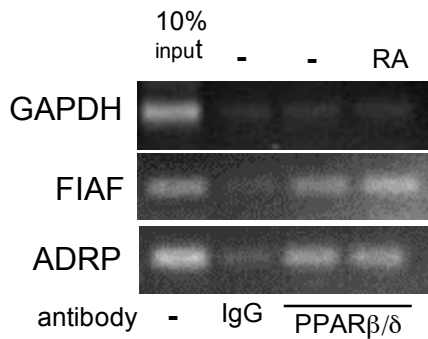


Figure 2.S1. PPAR β/δ associates with the PPRE of the ADRP and FIAF genes in live HaCaT cells. Chromatin immunoprecipitation assays were conducted in HaCaT cells using pre-immune IgG or antibodies against PPAR β/δ . The regions containing the PPRE of the *ADRP* and *FIAF* genes were amplified using appropriate primers (see Experimental Procedures). A 220 bp region 6-kb upstream from the start site of *GAPDH* was used as a negative control.

RA also enhanced the expression of the PPRE-driven reporter and did so in a dose-responsive manner (Fig. 2.2B). The response was markedly suppressed when the expression of PPAR β/δ in the cells was decreased by about 80% by siRNA methodology (Fig. 2.2B), indicating that the ability of RA to induce reporter expression was indeed mediated by this receptor and not by RAR.

We then set out to examine the ability of RA to induce the expression of endogenous PPAR β/δ target genes in HaCaT cells. One of these, *PDK-1*, was previously shown to comprise a direct PPAR β/δ target in HaCaT cells (Di-Poi et al., 2002). Two other genes, fasting induced adipose factor (*FIAF*) (Kersten et al., 2000) and adipose differentiation-related protein (*ADRP*) (Schmuth et al., 2004), were reported to be targeted by PPAR β/δ in other cells. Chromatin immunoprecipitation assays demonstrated that PPAR β/δ associates with the PPAR response elements of both *FIAF* and *ADRP* in HaCaT cells (Fig. 2.S1), verifying that both are direct targets for

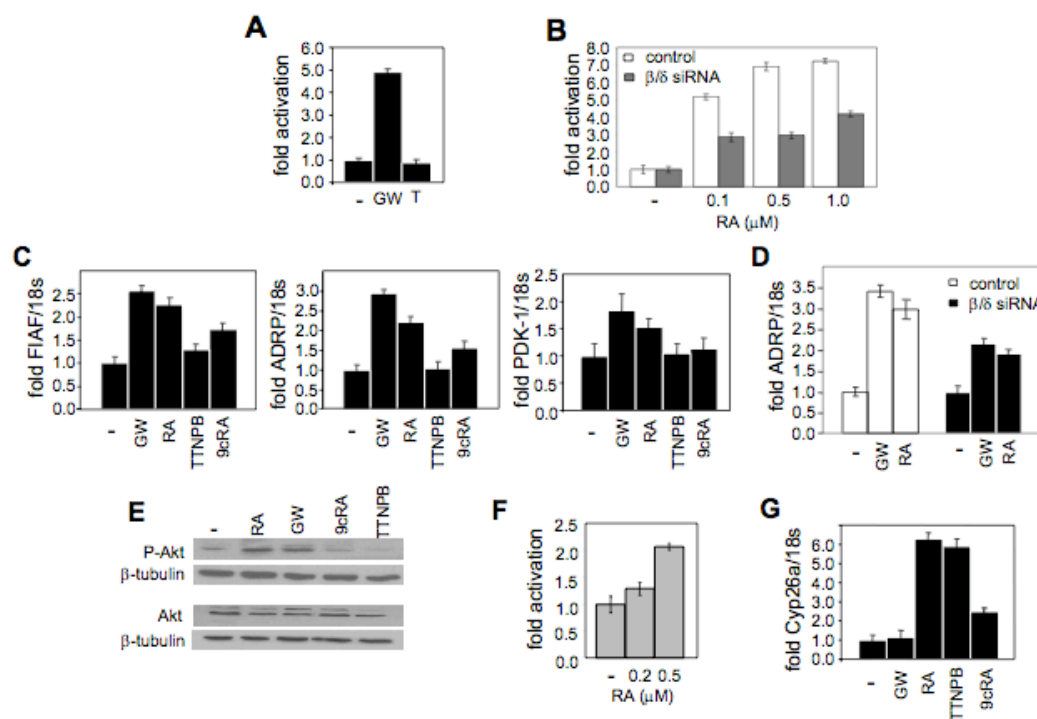


Figure 2.2. In HaCaT cells, RA activates both PPAR β/δ and RAR. **a. and b. A.** Cells were treated with vehicle or GW0742 (GW) or TTNPB (T, 1 μ M). **B.** Cells were cotransfected with either control siRNA or siRNA for PPAR β/δ , and then treated with RA at the denoted concentrations. **C.** HaCaT cells were treated with denoted ligands. Levels of mRNA of the PPAR β/δ target genes *FIAF*, *ADRP*, and *PDK-1* were analyzed by Q-PCR and normalized to 18s mRNA. Data are mean \pm SEM, n=3. **D.** HaCaT cells were transfected with control siRNA or PPAR β/δ siRNA (24 hr.), and then treated with the denoted ligands (0.1 μ M, 4 hr) and RNA extracted. **E.** Cells were treated with denoted ligands (0.1 μ M, 12 hr) and lysed. Thr-307-phospho-Akt, total Akt, and β -tubulin were assessed by immunoblots. **F.** Cells were treated with RA (15 hr), lysed, and luciferase activity measured and corrected for β -galactosidase activity. **G.** Cells were treated with the denoted ligands and expression of mRNA of the RAR target gene *Cyp26a* analyzed by Q-PCR. this receptor in the context of these cells.

The PPAR β/δ ligand GW0742 as well RA also enhanced the expression of the PPRE-driven reporter and did so in a dose-responsive manner (Fig. 2.2B). The response was markedly suppressed when the expression of PPAR β/δ in the cells was decreased by about 80% by siRNA methodology (Fig. 2.2B), indicating that the ability of RA to induce reporter expression was indeed mediated by this receptor and not by RAR.

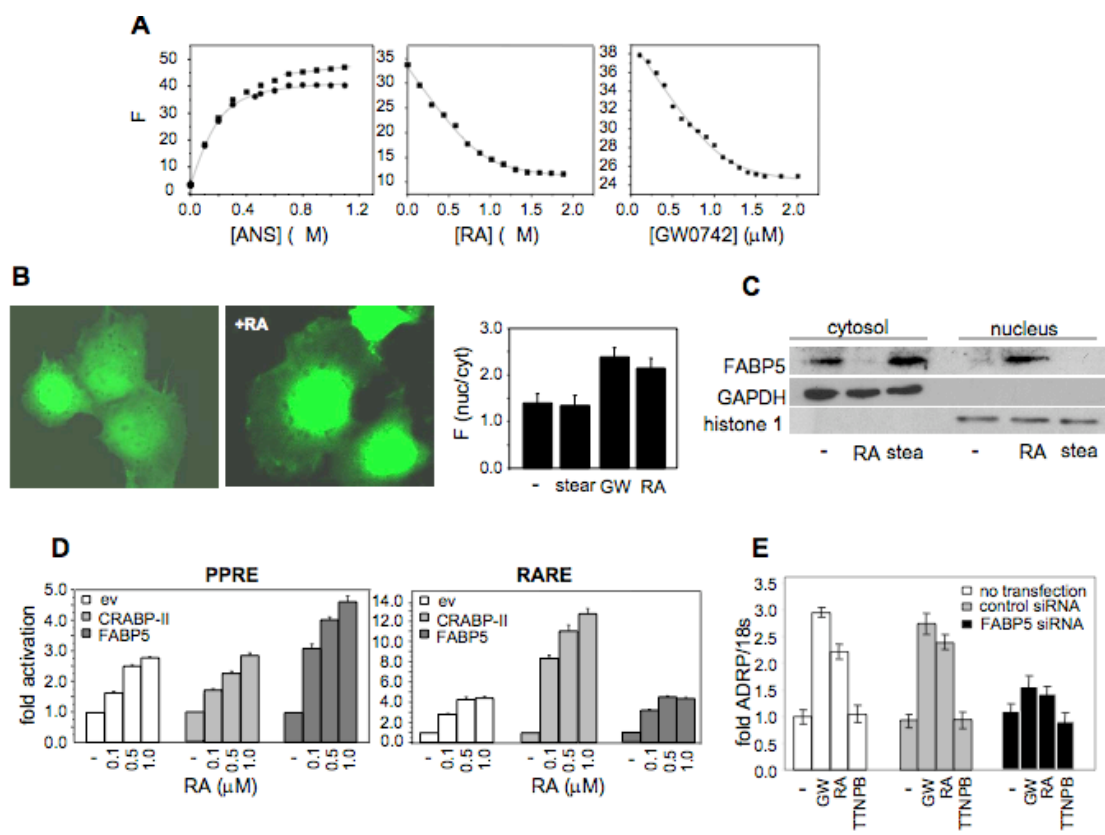
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Notably, one of the PPAR β/δ targets found to be induced by RA, PDK-1, is an important component of the anti-apoptotic activities of this receptor in keratinocytes, where induction of this kinase leads to phosphorylation and activation of the downstream PDK-1-target survival factor Akt (Di-Poi et al., 2002). The effects of RA or GW0742 on the phosphorylation level of Akt were thus examined. Treatment with either of these ligands, but not with TTNPB or 9cRA, significantly increased the phosphorylation level of Akt (Fig. 2.2E).

In addition to activating PPAR β/δ , RA also upregulated the expression of a reporter gene construct driven by an RAR response element (Fig. 2.2F), and it efficiently upregulated the expression of mRNA for *CYP26a*, a known direct RAR target gene (Loudig et al., 2000) (Fig. 2.2G). Hence, in HaCaT cells, RA treatment results in parallel activation of both RAR and PPAR β/δ .

FABP5 translocates into the nucleus in response to RA, and it enhances RA-induced, PPAR β/δ -mediated transcriptional activation. The observations that RA can activate both RAR and PPAR β/δ raise the question of the factors that regulate the dual activity of this hormone. We previously showed that the iLBPs CRABP-II and FABP5 mobilize to the nucleus in response to ligands that activate RAR and PPAR β/δ , respectively, and that they bind to their cognate receptors to form a complex through which the ligand is directly ‘channeled’ to the receptor. Consequently, CRABP-II enhances the transcriptional activity of RAR, while FABP5 facilitates the activation of PPAR β/δ (Budhu and Noy, 2002; Donato and Noy, 2005; Dong et al., 1999; Manor et al., 2003; Tan et al., 2002). The observations that RA serves as a ligand for PPAR β/δ thus raise the possibility that RA may be delivered to this receptor by FABP5. A fluorescence-based binding assay (Fig. 2.3A) demonstrated that GW0742 and RA bind to FABP5 with Kds of 42.3 ± 4.5 nM, and 34.8 ± 6.6 nM, respectively (mean \pm SD, n=3), in good agreement with binding

Figure 2.3. FABP5 binds RA, translocates to the nucleus in response to this ligand, and enhances RA-induced activation of PPAR β/δ . **A.** FABP5 was titrated with the fluorescence probe ANS. Titrations curves (left panel, filled squares) were corrected for linear non-specific fluorescence (solid line at end of titration curve), and corrected data (filled circles) analyzed to yield a K_d of 57 ± 7.3 nM (mean \pm SD, n=3). K_d s for the association of FABP5 with RA (middle panel) and with GW0742 (right panel) were determined by fluorescence competition titrations. **B.** COS-7 cells were transfected with an expression vector harboring GFP-FABP5 and imaged. Images were acquired from live cells before and after a 30 min. treatment with RA (1 μ M). Right panel: quantitation of nuclear/cytoplasmic partitioning of FABP5 in cells treated with denoted ligands. **C.** HaCaT cells were treated with denoted RA or with stearic acid (1 μ M, 30 min.). Nuclei were separated from cytosol by subcellular fractionation (Calbiochem ProtoExtract Subcellular Proteome Extraction kit) and analyzed for the presence of FABP5 by immunoblots. Efficiency of fractionation was validated by immunoblotting for the cytosolic marker GAPDH, and the nuclear marker histone 1. **D.** Transactivation assays were carried out in COS-7 cells cotransfected with a luciferase reporter driven by a PPRE and an expression vector for PPAR β/δ (left panel) or with an RARE-driven reporter together with an expression vector for RAR α (right panel). **E.** HaCaT cells were not transfected, or transfected with either control siRNA or a construct harboring FABP5 siRNA (24 hr.). The ability of denoted ligands to induce ADRP expression was monitored by Q-PCR and normalized to 18s mRNA. Data are mean \pm SEM, n=3.



affinities of this protein towards other ligands (Tan et al., 2002). The subcellular localization of FABP5 was then examined. COS-7 cells were transfected with FABP5 fused to green fluorescence protein (GFP), and confocal fluorescence microscopy was used to image GFP-FABP5 in live cells treated with various ligands (Fig. 2.3B). Similarly to the behavior of GFP when transfected alone, GFP-FABP5 in untreated cells distributed between the cytoplasm and the nucleus, most likely reflecting that over-expression of the protein leads to leakage into the nucleus even in the absence of a specific nuclear localization signal (data not shown and (Sessler and Noy, 2005). Treatment of cells with stearic acid, a long chain fatty acid that binds FABP5 but does not activate it, did not affect the subcellular distribution of the protein. In contrast, treatment with either GW0742 or RA resulted in a distinct shift of the protein into the nucleus (Fig. 2.3B). To monitor the effects of ligands on the localization of endogenous FABP5 in HaCaT cells, cells were treated with vehicle, RA, or stearate, subjected to subcellular fractionation, and the presence of FABP5 in cytosol and in nuclei examined by immunoblots (Fig. 2.3C). The data demonstrated that endogenous FABP5 in HaCaT cells is predominantly cytosolic in the absence of ligand, and that it accumulates in the nucleus in response to RA, but not upon treatment with stearic acid. Hence, like known PPAR β/δ -ligands, RA activates the nuclear localization of FABP5.

The effects of FABP5 on the ability of RA to activate PPAR β/δ were examined by transactivation assay using COS-7 cells which express very low level of either FABP5 or CRABP-II. Cells were co-transfected with a luciferase reporter construct driven by a PPRE, an expression vector for PPAR β/δ , and a vector harboring cDNA for either FABP5 or CRABP-II. Cells were then treated with RA, and the expression of the reporter monitored (Fig. 2.3D, left panel). RA enhanced the expression of the PPRE-driven reporter in a dose-responsive manner. While expression of CRABP-II did not affect the activity, FABP5 significantly enhanced

RA-induced, PPAR β/δ -mediated transactivation. To investigate the effect of the binding proteins on RA-induced activation of RAR, cells were transfected with a luciferase reporter under the control of an RAR response element (RARE), an expression construct for RAR α , and a vector harboring cDNA for either FABP5 or CRABP-II. In agreement with previous reports, CRABP-II augmented RA-induced transactivation of RAR. On the other hand, FABP5 had little effect on this activity (Fig. 2.3D, right pane). Cells in which the receptors were not ectopically expressed displayed qualitatively similar behaviour but the magnitudes of the ligand-induced responses were significantly smaller (not shown).

The involvement of FABP5 in RA-induced activation of PPAR β/δ was further investigated by examining the effect of decreasing the expression level of this binding protein on the ability of RA to activate the receptor in HaCaT cells. Cells were transfected with FABP5 siRNA, resulting in an about 80% decrease in the level of the protein, and induction of the PPAR β/δ target gene ADRP was monitored (Fig. 2.3E). Decreasing the expression of FABP5 markedly attenuated the ability of both GW0742 and RA to upregulate the expression of the ADRP, further substantiating that the presence of FABP5 is necessary for efficient activation of PPAR β/δ by its ligands, including RA.

Decreasing the FABP5/CRABP-II ratio in HaCaT and NaF cells converts RA from a survival factor to a pro-apoptotic agent. The observations that RA is delivered to RAR by CRABP-II, and is shuttled to PPAR β/δ by FABP5 suggest that differential expression profiles of these iLBPs may regulate the partitioning of RA between the two receptors in different cells. The levels of the two binding proteins were thus examined in several tissues and cells. Of special interest are mammary tumors that arise in the transgenic mouse cancer model MMTV*neu*. RA treatment of MMTV*neu* mice facilitates mammary tumor development (Fig. 2.1). On the other

hand, it was previously reported that ectopic expression of CRABP-II in these tumors induces carcinoma cell apoptosis and suppresses tumor growth (Manor et al., 2003), indicating that, under these conditions, RA exerts growth-inhibitory activities. Comparison between the expression levels of CRABP-II and FABP5 in tumors that arise in MMTV*neu* mice and in adjacent normal mammary tissue revealed that CRABP-II expression is markedly decreased, while the level of FABP5 is significantly higher in the tumors (Fig. 2.4A). Hence, tumor development in MMTV*neu* mice is accompanied by a dramatic decrease in the CRABP-II/FABP5 ratio. Corresponding to this expression profile *in vivo*, NaF cells, a cell line derived from MMTV*neu* tumors, display a high level of FABP5 and minimal expression of CRABP-II (Fig. 2.4B). Similarly, HaCaT cells, in which RA can function through PPAR β/δ , express a high level of FABP5 and an undetectable CRABP-II content (Fig. 2.4B). In contrast, the mammary carcinoma MCF-7 cells, in which RA activates RAR to inhibit cell growth (Donato and Noy, 2005; Donato et al., 2007; Mangiarotti et al., 1998; Toma et al., 1998), and F9 teratocarcinoma cells, which differentiate into primitive endoderm in response to RA-induced, RAR-mediated signalling (Rochette-Egly and Chambon, 2001; Strickland and Mahdavi, 1978; Strickland et al., 1980), express a markedly higher CRABP-II/FABP5 ratio (Fig. 2.4B).

The effect of reversing the CRABP-II/FABP5 ratio in HaCaT cells on the ability of RA to activate PPAR β/δ was then examined. Cells were transfected with either an expression vector for CRABP-II, or with a construct harboring FABP5 siRNA (Fig. 2.4C), treated with RA or GW0742, and induction of the PPAR β/δ target gene PDK-1 was monitored (Fig. 2.4D). Over-expression of CRABP-II had little effect on GW0742-induced expression of PDK-1 mRNA, indicating that this protein does not directly affect PPAR β/δ activity. Either over-expression of CRABP-II or under-expression of FABP5 abolished the ability of RA to induce PDK-1 expression. Additionally, in accordance with the observations that

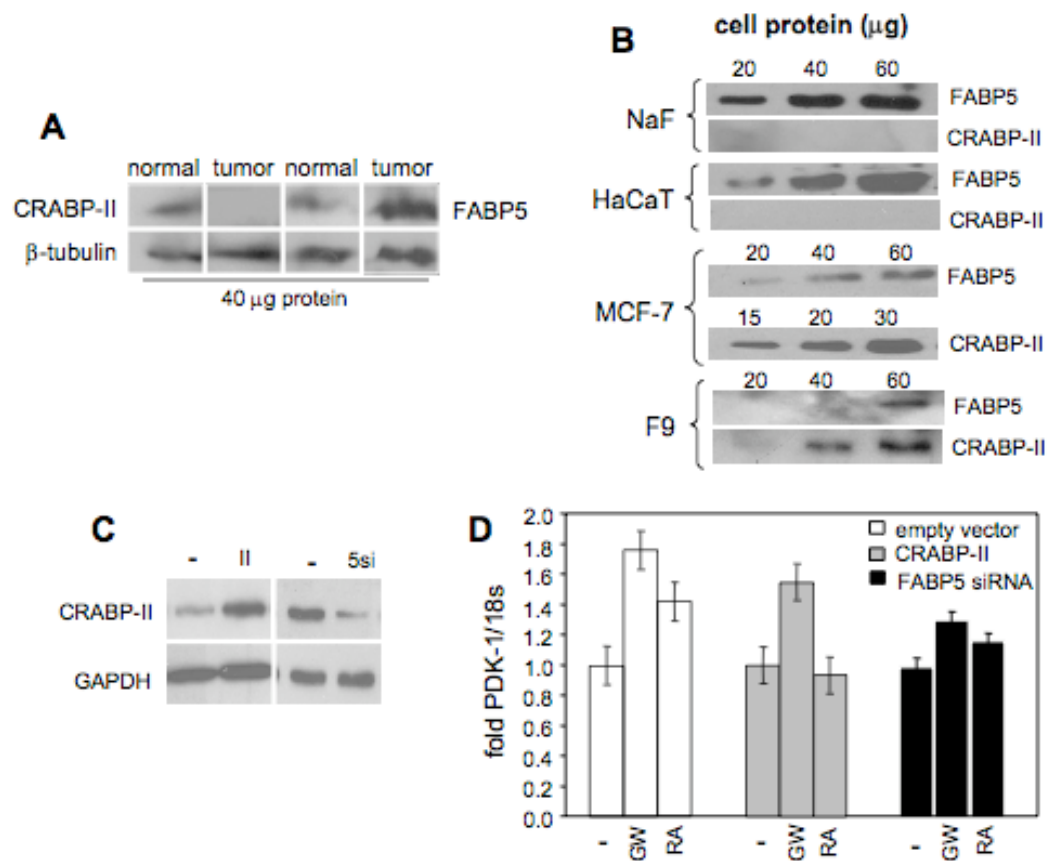


Figure 2.4. In HaCaT cells, decreasing the FABP5/CRABP-II ratio diverts RA away from PPAR β/δ . **A.** Expression of CRABP-II and FABP5 in mammary tumors that arise in MMTV*neu* mice and in adjacent normal mammary tissue estimated by immunoblots. **B.** Expression levels of CRABP-II and FABP5 in HaCaT, NaF, MCF-7, and F9 cells were estimated by immunoblots. **C.** HaCaT cells were transfected with an expression vector for CRABP-II or with a vector harboring FABP5 siRNA. Effectiveness of transfection in modulating the proteins' expression levels was monitored by immunoblotting. **D.** Parental HaCaT cells and HaCaT cells over-expressing CRABP-II or under-expressing FABP5 were treated with denoted ligands (1 μ M, 4 hr) and expression of PDK-1 mRNA was measured by Q-PCR. Data are mean \pm SEM, n=3.

GW0742 tightly binds to FABP5 and mobilizes it to the nucleus (Fig. 2.3), decreasing

FABP5 expression augmented the transcriptional activity of this ligand. The data thus show that increasing the CRABP-II/FABP5 ratio in HaCaT cells diverts RA away from PPAR β/δ . The involvement of FABP5 in induction of PDK-1 by both RA and GW0742 also supports the notion that this binding protein mediates the nuclear targeting of both of these PPAR β/δ ligands.

Upregulation of PDK-1 expression upon activation of PPAR β/δ by RA in HaCaT cells results in the phosphorylation and thus the activation of the survival factor Akt (Fig. 2.2E). It can thus be predicted that RA treatment will protect these cells against apoptosis. To examine this notion, HaCaT cells were treated with the apoptosis inducer tumor necrosis factor α (TNF α), and the effects of RA, the PPAR β/δ ligand GW0742, and the RAR ligand TTNPB on TNF α -induced apoptosis were examined. To this end, we monitored the ability of TNF α to trigger the cleavage of poly(ADP-ribose) polymerases (PARP), one of the earliest proteins targeted for a specific cleavage during apoptosis (Fig. 2.5A, 5B). TNF α treatment significantly enhanced PARP cleavage, demonstrating the efficacy of the cytokine as an apoptosis-inducing agent in these cells. In agreement with a role for PPAR β/δ in enabling keratinocyte survival during inflammation (Di-Poi et al., 2002; Tan et al., 2001), activation of the receptor by GW0742 inhibited TNF α -induced apoptosis. Similarly to GW0742, RA protected the cells from TNF α -induced apoptosis. In contrast, the RAR-ligand TTNPB induced apoptosis when administered alone, and augmented TNF α -induced PARP cleavage, demonstrating that RAR displays pro-apoptotic activities in these cells. Hence, in HaCaT cells, RA inhibits apoptosis like a *bona-fide* PPAR β/δ ligand, an activity that diametrically contrasts with the pro-apoptotic activities of RAR.

The hypothesis that CRABP-II and FABP5 control the partitioning of RA

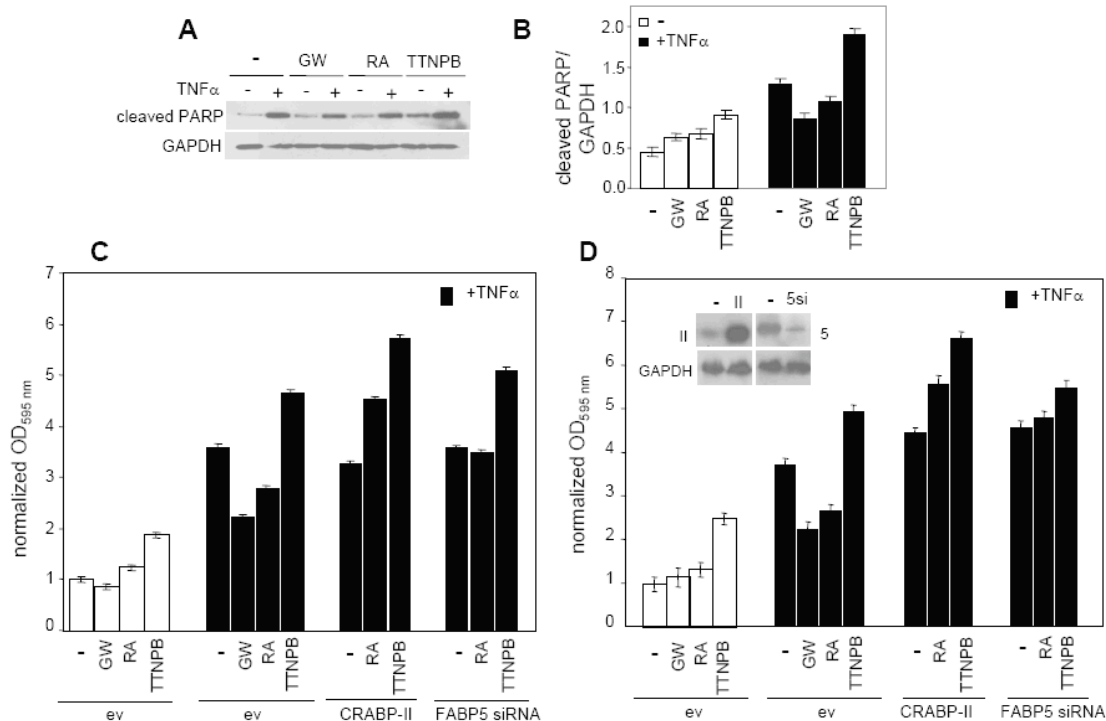


Figure 2.5. In HaCaT and NaF cells decreasing the FABP5/CRABP-II ratio converts RA from an anti-apoptotic to a pro-apoptotic agent. **A.** HaCaT cells were treated with denoted ligands (2 μ M, 2 hr) prior to addition of TNF α (20 ng/ml, 14 hr.). Cells were lysed and the level of cleaved PARP assessed by immunoblots. A representative experiment is shown. **B.** Quantitation of data described in panel **a**. Band intensities were normalized to the loading control GAPDH. Data are mean \pm SEM, n=3. **C.** and **D.** Parental HaCaT (**C.**) or NaF (**D.**) cells and corresponding cells over-expressing CRABP-II or under-expressing FABP5 were treated with denoted ligands (1 μ M, 16 hr.), or pretreated with ligands for 2 hr. prior to addition of TNF α (20 ng/ml, 14 hr., solid bars). Apoptosis was evaluated using the APOPercentage Apoptosis Assay kit. Data are mean \pm SEM, n=3.

between RAR and PPAR β/δ and the biological activities of this hormone, was further investigated by examining the outcome of changing the CRABP-II/FABP5 ratio on the ability of RA to modulate TNF α -induced apoptosis. In these experiments,

apoptosis was followed by monitoring the transfer of phosphatidylserine to the outer leaflet of the cell membrane, a process linked to the execution phase of apoptosis. In agreement with observations obtained by monitoring PARP cleavage as a ‘readout’ for apoptosis, treatment of HaCaT cells with GW0742 or RA had little effect, while the RAR-ligand TTNPB increased the fraction of apoptotic cells (Fig. 2.5C). Also in agreement, TNF α efficiently induced apoptosis, both GW0742 and RA inhibited, and TTNPB enhanced the response. Strikingly, over-expression of CRABP-II not only abolished the protective activity of RA, but converted it into an agent which, similarly to TTNPB, enhanced TNF α -induced apoptosis. Reduced expression of FABP5 also negated the anti-apoptotic activity of RA. The observation that the decreased expression of FABP5 did not quite lead to an enhancement of the apoptotic response likely reflects that transfection of FABP5 siRNA reduced but did not completely inhibit the expression of the protein (Fig. 2.4C). Similar effects were observed in NaF cells, derived from mammary tumors of the MMTV neu mouse model; RA ‘rescued’ NaF cells from TNF α -induced apoptosis, and either over-expression of CRABP-II or under-expression of FABP5 abolished the protective activity of RA and enhanced the apoptotic response (Fig. 2.5D).

In MCF-7 cells, increasing the FABP5/CRABP-II ratio converts RA from a pro-apoptotic to an anti-apoptotic agent. Unlike HaCaT and NaF cells, the mammary carcinoma MCF-7 cells display a high CRABP-II/FABP5 ratio (Fig. 2.4B). To reverse this ratio, cells were co-transfected with an expression vector for FABP5 and a construct harboring CRABP-II siRNA (Fig. 2.6A). The ensuing concomitant increase in FABP5 and decrease in CRABP-II levels did not affect the expression of either RAR or PPAR β/δ (Fig. 2.S2). Nevertheless, the reversal hampered the ability of RA to upregulate the expression of the cell cycle regulator *BTG2*, a gene that was recently shown to comprise a direct target for RAR and to be involved in mediating RA-

induced cell cycle arrest in MCF-7 cells (Donato et al., 2007)(Fig. 2.6B). Correspondingly, the reversal abolished the ability of RA to down-regulate the expression of cyclin D1, a known down-stream target for BTG2 (Guardavaccaro et al., 2000; Kawakubo et al., 2006; Kawakubo et al., 2004; Lim et al., 1989) (Fig. 2.6C). Reversing the FABP5/CRABP-II also inhibited RA-induced induction of the RAR target cyp26a (Fig. 6D). Remarkably, while RA had little effect on the level of the direct PPAR β/δ target gene PDK-1 in parental MCF-7 cells, the ligand significantly upregulated the expression of this survival factor upon the reversal of the ratio of the binding proteins (Fig. 2.6E). Hence, increasing the FABP5/CRABP-II ratio in MCF-7 cells directed RA away from RAR and towards PPAR β/δ .

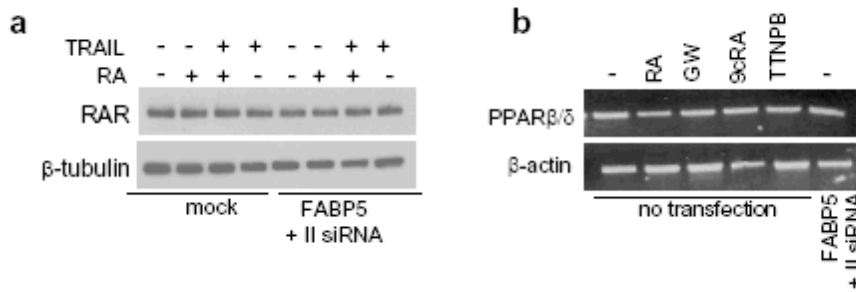


Figure 2.S2. Switching the FABP5/CRABP-II ratio in MCF-7 cells does not affect the expression of either RAR or PPAR β/δ . MCF-7 cells were mock transfected or co-transfected with an expression vector for FABP5 and with CRABP-II siRNA, and treated as denoted. **a.** The level of all RAR isotypes was assessed by immunoblots using pan-RAR antibodies (Santa Cruz Biotechnology). **b.** The levels of mRNA for PPAR β/δ were measured by Q-PCR using TaqMan chemistry and Assays on Demand probes (Applied Biosystems, Hs00602622_m1), and normalized to 18s.

Considering the disparate nature of the RAR and PPAR β/δ target genes, the observations suggest that the reversal of the CRABP-II/FABP5 ratio hampered growth-inhibitory activities and triggered a survival response.

To further examine the consequences of the ‘switch’ in binding protein profile, parental MCF-7 cells and cells in which the CRABP-II/FABP5 ratio was reversed were treated with the apoptosis-inducing agent tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Apoptosis was monitored by following PARP cleavage (Fig. 2.6F) as well as by monitoring transfer of phosphatidylserine to the outer leaflet of the cell membrane (Fig. 2.6G). Transfection of constructs encoding FABP5 and CRABP-II siRNA resulted in PARP cleavage even in the absence of TRAIL. The effect is likely to stem from a degree of apoptosis induced by the forced over-expression, and was not observed upon monitoring later apoptosis events, represented by phosphatidylserine membrane “flip”. Treatment with TRAIL induced marked apoptosis as judged by both PARP cleavage and by plasma membrane events. Addition of TRAIL in conjunction with RA had little effect (PARP cleavage) or somewhat enhanced apoptosis (phosphatidylserine translocation). Remarkably, both ‘read-outs’ clearly demonstrated that, in cells in which the CRABP-II/FABP5 ratio was reversed, RA treatment markedly inhibited TRAIL-induced apoptosis. Hence, increasing the FABP5/CRABP-II ratio in MCF-7 cells results in conversion of RA from a growth-inhibitory to a pro-survival factor

2.5 Discussion

RA activates the nuclear receptor RAR and, in many embryonic and adult tissues, the biological activities of this hormone can be traced to induction of expression of RAR target genes. However, in some tissues, such as skin, important functions of RA appear to be mediated by an RAR-independent pathway, the nature of which

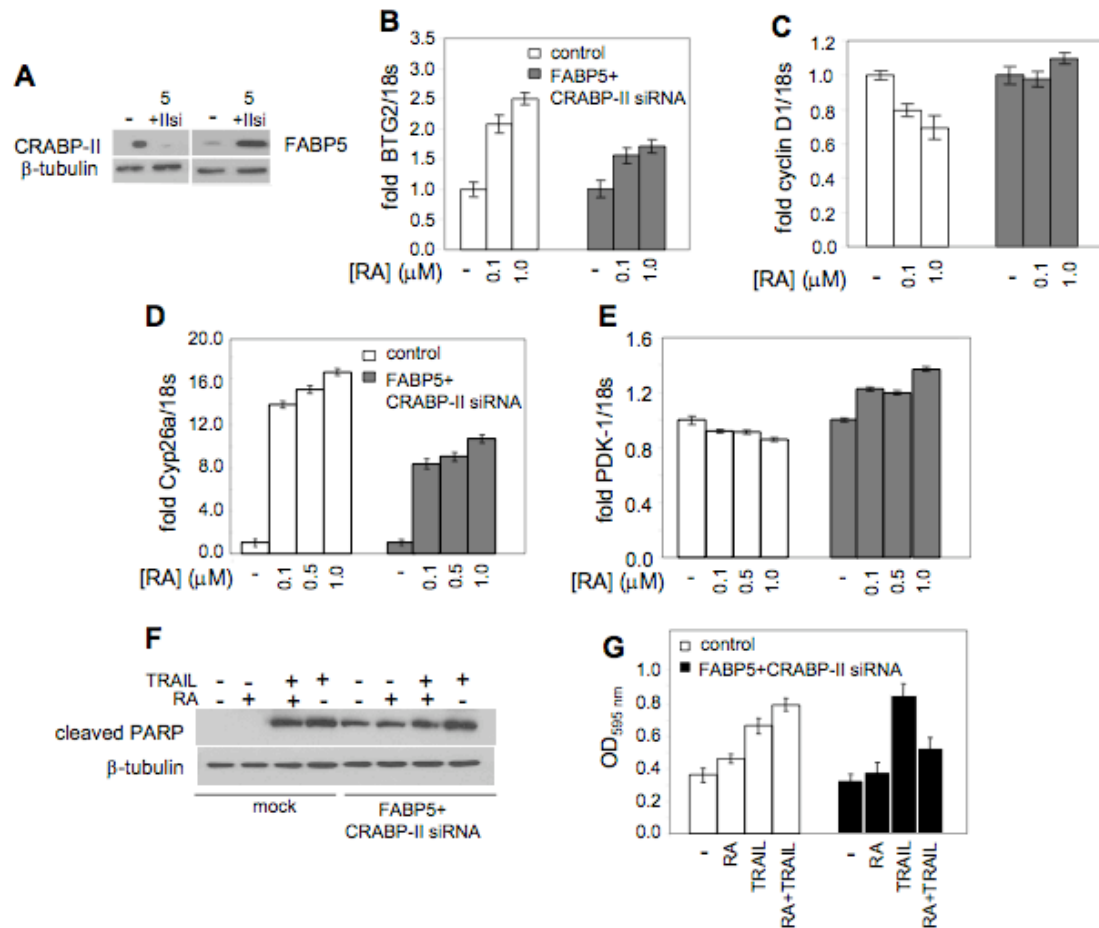


Figure 2.6. In MCF-7 cells, increasing the FABP5/CRABP-II ratio converts RA from a pro-apoptotic agent to a survival factor. **A.** MCF-7 cells were co-transfected with an expression vector for FABP5 together with CRABP-II siRNA, and protein expression levels monitored by immunoblotting. **B.-E.** MCF-7 cells were mock transfected or co-transfected with an expression vector for FABP5 and with CRABP-II siRNA, and treated with RA. Levels of mRNA for BTG2 (**B.**), cyclin D1 (**C.**), cyp26a (**D.**), and PDK-1 (**E.**) were measured by Q-PCR. **F.** and **G.** MCF-7 cells were mock transfected or co-transfected with an expression vector for FABP5 and with CRABP-II siRNA. Cells were treated with TRAIL (4 ng/ml), or RA (0.1 μM), or both, and apoptosis was assessed (**F.**) and by monitoring transfer of phosphatidylserine using the APOPercentage Apoptosis Assay kit (**G.**).

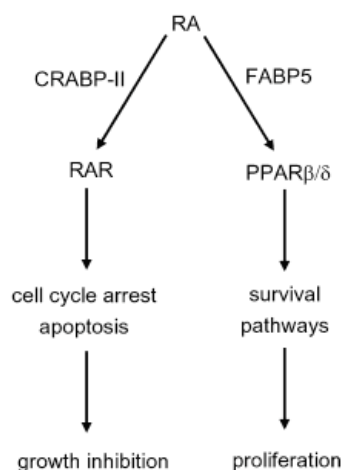


Figure 2.7. A model outlining the dual transcriptional activity of RA. CRABP-II and FABP5 target RA to RAR and PPAR β/δ , respectively. In cells that express a high CRABP-II/FABP5 ratio, RA is ‘channeled’ to RAR, often resulting in growth inhibition. Conversely, in the presence of a low CRABP-II/FABP5 expression ratio, RA is targeted to PPAR β/δ , thereby upregulating survival pathways.

remained unclear. The observations presented here indicate that, in addition to activating RAR, RA can also activate the nuclear receptor PPAR β/δ , and thus that the repertoire of genes and cellular responses that can be controlled by this hormone include both RAR and PPAR β/δ -targets. The data show further that partitioning of RA between its two receptors is regulated by cognate iLBPs; CRABP-II delivers RA to RAR, while FABP5 shuttles the hormone to PPAR β/δ (Fig.2.7). It should be noted that the binding affinity of the CRABP-II/RAR pathway for RA exceeds that of the FABP5/PPAR β/δ path. The interactions of RA with both CRABP-II and RAR are characterized by Kds in the 0.1-0.2 nM range (Dong et al., 1999; Sussman and de Lera, 2005), while both FABP5 and PPAR β/δ associate with this compound at a Kd

of 10-50 nM ((Tan et al., 2002) and Fig. 3A). It can thus be predicted that, in most cells, RA signalling through RAR will predominate, and that activation of PPAR β/δ will become apparent only in cells that exhibit a high FABP5/CRABP-II ratio. This indeed appears to be the case. In MCF-7 cells, which express a low FABP5/CRABP-II ratio, RA activates RAR, while keratinocytes and NaF cells, which display a high FABP5/CRABP-II ratio, respond to RA by activation of PPAR β/δ .

A high FABP5/CRABP-II ratio abolishes RA-triggered upregulation of RAR target genes that mediate important biological responses, such as cell cycle arrest (Fig. 2.6B, 2.6C). It is worth noting however that cells that display such a ratio retain the ability to induce the expression of very efficient RAR targets, such as the RA-degrading enzyme Cyp26a, albeit at a muted response (Fig. 2.2G and 2.6D). Hence, critical functions of RA that are mediated by RAR, such as the ability to trigger its own degradation by upregulating the expression of cyp26 (White et al., 1996), can proceed in the background of the predominant action of RA-evoked PPAR β/δ signalling.

The present work demonstrates that RA serves as a physiological ligand for PPAR β/δ under some but not all circumstances. However, this receptor displays near-ubiquitous tissue expression (Kliewer et al., 1994), raising the question of the nature of the ligand(s) that activate it in tissues that do not support activation by RA. The ligand-binding pocket of PPAR β/δ is much larger than the pockets of other nuclear receptors (Xu et al., 1999). It may thus accommodate multiple ligands, and it has been suggested that various long chain fatty acids and eicosanoids may serve as effective PPAR β/δ activators (Bucco et al., 1997). Whether some of these ligands function as true physiological ligands for the receptor remains to be clarified, but the present work and the similar nature of ligands that bind to FABPs and PPARs (Hanhoff et al., 2002; Widstrom et al., 2001) raise the possibility that FABPs other than FABP5 may act to deliver ligands other than RA to PPAR β/δ , and thus that they

may regulate the functionality of distinct ligands in specific tissues.

When enabled, RA signalling through PPAR β/δ has profound functional consequences. One consequence, explored here, is that such signalling evokes potent anti-apoptotic activities that overcome the growth-inhibitory activities of RAR, and allow cells to survive in the face of powerful apoptotic agents. Hence, RA-dependent maintenance of skin integrity, proliferation of basal keratinocytes, and survival of these cells during wound repair likely stem from a high expression level of FABP5, enabling RA to activate PPAR β/δ . Activation of PPAR β/δ by RA also appears to underlie the facilitation of mammary tumor progression in MMTV*neu* mice by this hormone. In support of this conclusion are the observations that RA functions as anti-apoptotic agent in cells derived from MMTV*neu* tumors, that a high FABP5/CRABP-II ratio is required for this activity, that tumor progression in MMTV*neu* mice is accompanied by marked increase in the FABP5/CRABP-II ratio, and that decreasing this ratio by ectopic over-expression of CRABP-II triggers apoptosis and inhibits tumor development *in vivo* (Manor et al., 2003). It is worth noting in regard to this that it has been reported FABP5 is expressed in melanocytic tumors, but not in normal human melanocytes (Brouard et al., 2002), indicating that elevation in the expression of this protein, and thus activation of PPAR β/δ accompanies tumorigenesis in various cancers. While not directly addressed here, the observations that RA is critical for neuronal survival (Henion and Weston, 1994; Jacobs et al., 2006; Plum et al., 2001; Rodriguez-Tebar and Rohrer, 1991; Wuarin et al., 1990) raise the possibility that the brain may comprise another organ in which RA signals through PPAR β/δ . It is interesting to note regarding this possibility that the expression level FABP5 is high during neurogenesis in the developing rat brain (Liu et al., 2000), that this protein is required for neuronal outgrowth in PC12 neuronal cells (Allen et al., 2000), and that its expression is induced following nerve injury (De Leon et al., 1996), suggesting an involvement in

survival/repair processes.

2.6 Acknowledgements

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CHAPTER THREE

Overcoming retinoic acid-resistance of mammary tumors by diverting retinoic acid from PPAR β/δ To RAR

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3.1 Summary

Retinoic acid (RA) displays potent anticarcinogenic activities which are mediated by the nuclear receptor RAR. However, in some cancers, RA facilitates tumor growth. It was recently suggested that this paradoxical activity may be mediated by another receptor, PPAR β/δ , and that partitioning of RA between its two receptors is regulated by CRABP-II and FABP5, which target RA to RAR and PPAR β/δ , respectively. Here, we generated *MMTV-neu* mammary carcinoma mouse models that express varying mammary CRABP-II/FABP5 ratios. We show that, in neoplasms of *MMTV-neu* mice, RA does not activate RAR, but activates PPAR β/δ to enhance proliferation. Increasing the CRABP-II/FABP5 ratio diverts RA to RAR, thereby markedly suppressing tumor growth. The data demonstrate the existence of a novel mechanism that underlies RA-resistance in tumors.

3.2 Significance

Despite promising clinical results, use of RA in oncology is limited by RA-resistance acquired during carcinogenesis. RA inhibits tumor growth by activating the nuclear receptor RAR, but the basis for development of RA-resistance remains incompletely understood. We show that, in the RA-resistant mouse model of breast cancer *MMTV-neu*, RA does not activate RAR but, instead, activates the ‘non-classical’ RA-receptor PPAR β/δ , thereby enhancing proliferation. This behavior was traced to aberrant expression of CRABP-II and FABP5, which target RA to RAR and PPAR β/δ , respectively. Increasing the CRABP-II/FABP5 ratio in mammary tissue diverted RA from PPAR β/δ to RAR and suppressed tumor growth. Hence, CRABP-II is a tumor suppressor, and inhibition of FABP5 may comprise a novel therapeutic strategy for overcoming RA-resistance in tumors.

3.3 Introduction

The vitamin A metabolite all-*trans*-retinoic acid (RA) is a potent inhibitor of carcinogenesis and is currently used as a therapeutic agent in several types of human cancer (Altucci and Gronemeyer, 2001; Soprano et al., 2004). The anti-carcinogenic activities of this hormone are mediated by retinoic acid receptors (RAR α , β , and γ), members of the nuclear receptor family of ligand-activated transcription factors. Like other subclass 1 nuclear receptors, RARs interact with the retinoid X receptor (RXR) to form heterodimers that bind to DNA through RAR response elements in regulatory regions of specific target genes (Chambon, 1996; Kitareewan et al., 2002). In the absence of RA, DNA-bound RAR associates with a corepressor complex which includes components that catalyze histone deacetylation, leading to a compact chromatin structure and transcriptional repression (Xu et al., 1999). The first step in activation of the receptor entails delivery of RA from the cytosol to RAR in the nucleus. This step is mediated by cellular retinoic acid binding protein II (CRABP-II), a cytosolic protein that, upon binding RA, mobilizes to the nucleus, where it directly interacts with RAR to form a complex through which RA is ‘channeled’ to the receptor (Budhu et al., 2001; Budhu and Noy, 2002; Dong et al., 1999; Sessler and Noy, 2005). Upon binding of RA, the C-terminal of RAR undergoes a large conformational change, leading to dissociation of corepressors and recruitment of a coactivator complex (Moras and Gronemeyer, 1998). In turn, coactivators catalyze histone acetylation to loosen chromatin structure and enable transcription to proceed (Xu et al., 1999). A different class of coactivators, termed Mediators, associate with liganded RAR and ‘bridge’ between the receptor and the general transcription machinery (Rachez and Freedman, 2001).

Once activated, RAR upregulates the expression of multiple target genes including genes involved in differentiation (Park et al., 1999; Rochette-Egly and Chambon, 2001), cell cycle control (Donato et al., 2007), and apoptosis (Donato and

Noy, 2005; Gumireddy et al., 2003; Oltersdorf et al., 2005; Srivastava et al., 1999). Activated RAR thus often inhibits cell growth and it displays distinct anticarcinogenic activities (Altucci and Gronemeyer, 2001; Soprano et al., 2004). However, despite increased understanding of the mechanisms by which RA inhibits cancer cell growth and promising pre-clinical and clinical results, use of retinoids in cancer therapy and prevention remains limited. Such therapy is hampered both by pronounced toxicity of RA when administered at pharmacological doses, and by the development of RA-resistance during carcinogenesis (Garattini et al., 2007; Mongan and Gudas, 2007). It has been demonstrated that RA-resistance may stem from suppression of various steps of the overall process of transcriptional activation by the hormone. Hence, in different carcinomas, such resistance has been reported to involve defects in RA synthesis (Arapshian et al., 2000), down-regulation of CRABP-II (Budhu and Noy, 2002; Jing et al., 1997), loss of expression of RAR, in particular through epigenetic silencing of RAR β 2 (Olasz et al., 2007; Xu, 2007), and impaired ligand-induced corepressor/coactivator exchange (Slack, 1999). Accordingly, it has been demonstrated that RA-responsiveness may be restored to carcinomas by targeting specific repressed steps, e.g. by ectopic expression of CRABP-II (Budhu and Noy, 2002; Manor et al., 2003) or RAR β 2 (Campbell et al., 1998; Hoffman et al., 1996), and by chemical inhibition of histone deacetylases (He et al., 2001; Warrell et al., 1998).

Notably, various reports indicated that some carcinomas not only display “RA-resistance”, i.e. a failure to become growth-inhibited upon treatment with the agent, but, instead, administration of RA to these cells confers enhanced proliferation. For example, it was reported that RA treatment facilitates skin tumor formation (Verma et al., 1982), enhances tumor growth in the MMTV-*neu* mouse model of breast cancer (Schug et al., 2007), augments proliferation induced by epidermal growth factor in cultured explants of mouse mammary gland (Komura et al., 1986),

and, under some circumstances, promotes at late stages of malignant progression (Sapi et al., 1999). In addition, the β -Carotene and Retinol Efficacy Trial (CARET), a multicenter lung cancer chemoprevention trial with smoker and asbestos-exposed populations was terminated in 1996, 21 months ahead of schedule, due to the findings that the treatment resulted in 28% increase in lung cancer incidence and in 46% higher mortality rate from lung cancer (Omenn et al., 1996). While these effects may have potentially stemmed from RA-independent activities or retinol or β -carotene, the observations support the notion that, under some conditions, retinoids possess procarcinogenic activities. Such activities are unlikely to be mediated by RAR. Proliferative, RAR-independent activities of RA were also suggested by the observations that, although this hormone plays critical roles in maintenance of skin integrity, all RAR subtypes are dispensable for keratinocyte renewal (Chapellier et al., 2002).

A clue as to a possible basis for the RAR-independent activities of RA was recently provided by the observations that this hormone also serves as a ligand for another nuclear receptor, namely, PPAR β/δ . It was thus demonstrated that RA binds to PPAR β/δ with a high affinity, and that it induces the ability of the receptor to transactivate reporter gene constructs as well as endogenous target genes (Schug et al., 2007; Shaw et al., 2003). In view of the reports that target genes for PPAR β/δ include genes involved in proliferative responses, such as VEGF (Wang et al., 2006), and genes that activate survival pathways, e.g. PDK1 (Di-Poi et al., 2002; Icre et al., 2006; Tan et al., 2004), it was suggested that activation of this receptor by RA may underlie pro-proliferative activities of the hormone. It was shown in regard to this that RA-triggered activation of PPAR β/δ in keratinocytes protects these cells against apoptosis triggered by the potent apoptotic agent TNF α (Schug et al., 2007).

The observations that RA can activate both RAR and PPAR β/δ raise the question of the mechanism that regulate the partitioning of the hormone between the

two receptors. The key to this transcriptional targeting was found to lie with two intracellular lipid-binding proteins: CRABP-II which delivers RA to RAR (Budhu et al., 2001; Budhu and Noy, 2002; Dong et al., 1999; Sessler and Noy, 2005), and FABP5 (K-FABP, eFABP, mal1), a CRABP-II-homolog which targets the hormone to PPAR β/δ (Schug et al., 2007; Tan et al., 2002). Similarly to CRABP-II, FABP5 mobilizes to the nucleus upon binding of RA but, while CRABP-II delivers RA to RAR, FABP5 channels the ligand to PPAR β/δ (Schug et al., 2007). Indeed, it was demonstrated that RA signals through RAR and inhibits the growth of cells that express a high level of CRABP-II, but functions predominately through PPAR β/δ and displays anti-apoptotic activities in cells that contain a high FABP5/CRABP-II ratio (Schug et al., 2007).

These observations suggest that the RA-resistance of some tumors may result from targeting of RA to PPAR β/δ rather than to RAR. To examine this hypothesis, we used the FVB/N-Tg(*MMTVneu*)202*Mul/J* (*MMTV-neu*) transgenic mouse model of breast cancer. In this model, *Neu/Erb-B2/Her2*, which encodes for a member of the epidermal growth factor receptor protein tyrosine kinase family, is specifically over-expressed in the mammary epithelium under the control of *MMTV-LTR* promoter/enhancer, resulting in spontaneous development of mammary tumors in 100% of female mice (Guy et al., 1992). Amplification of this gene has been observed in a significant proportion of primary human breast cancers (King et al., 1985; Yokota et al., 1986), and a strong correlation has been established between the extent of overexpression and poor outcome in human patients (Slamon et al., 1989). Notably, over-expression of *Neu* induces profound RA-resistance in cultured cells (Tari et al., 2002) as well as *in vivo* (Schug et al., 2007). The basis for this resistance is currently unknown. However, the observations that carcinogenesis in *MMTV-neu* mice is accompanied by an increase in the FABP5/CRABP-II ratio in tumors (Schug et al., 2007), and that administration of an adenovirus encoding CRABP-II to these

tumors suppresses their growth (Manor et al., 2003) raise the possibility that the RA-resistance of these tumors originates from deregulation of the expression ratio of the two RA-binding proteins, which, in turn, may lead to RA signalling through PPAR β/δ rather than through RAR. To examine this hypothesis, we generated *MMTV-neu* mice models with varying mammary FABP5/CRABP-II ratios and investigated the transcriptional activities of RA and the outcomes of these activities for tumor development in these mice.

3.4 Results

Generation of transgenic mice with varying mammary FABP5/CRABP-II ratios.

To explore the possibility that changes in the expression ratio of CRABP-II and FABP5 determine cellular responses to RA signaling *in vivo*, we generated two new *MMTV-neu* mammary carcinoma mouse models. One of these consisted of *MMTV-neu* mice in which expression of CRABP-II is disrupted, leading to a very high FABP5/CRABP-II ratio. This model was established by crossing *MMTV-neu* mice with CRABP-II-null mice (Lampron et al., 1995), resulting in *MMTV-neu*^{+/-}/*CRABP-II*^{-/-} mice (termed MCRABP-II^{-/-} in this manuscript). (see Experimental Procedures for details of crosses).

A second model entailed *MMTV-neu* mice that specifically over-express CRABP-II in mammary tissue and thus display a low FABP5/CRABP-II ratio. Such mice were generated using a transgenic construct consisting of the mammary epithelium-specific promoter/enhancer MMTV-LTR, derived from the mouse mammary tumor virus, a synthetic human CRABP-II cDNA construct, and a human beta globin polyadenylation signal (Fig 3.1a). Transgenic founders were identified by PCR, and gene integration confirmed by Real Time quantitative PCR (Q-PCR). Mammary expression of the transgene was verified by immunoblotting, and by Q-PCR analysis of mammary glands and other tissues (Fig 3.1b-3.1d). These mice were

crossed with *MMTV-neu* mice to generate *MMTV-neu*^{+/−}/*MMTV-CRABP-II* animals (termed here MTgCRABP-II).

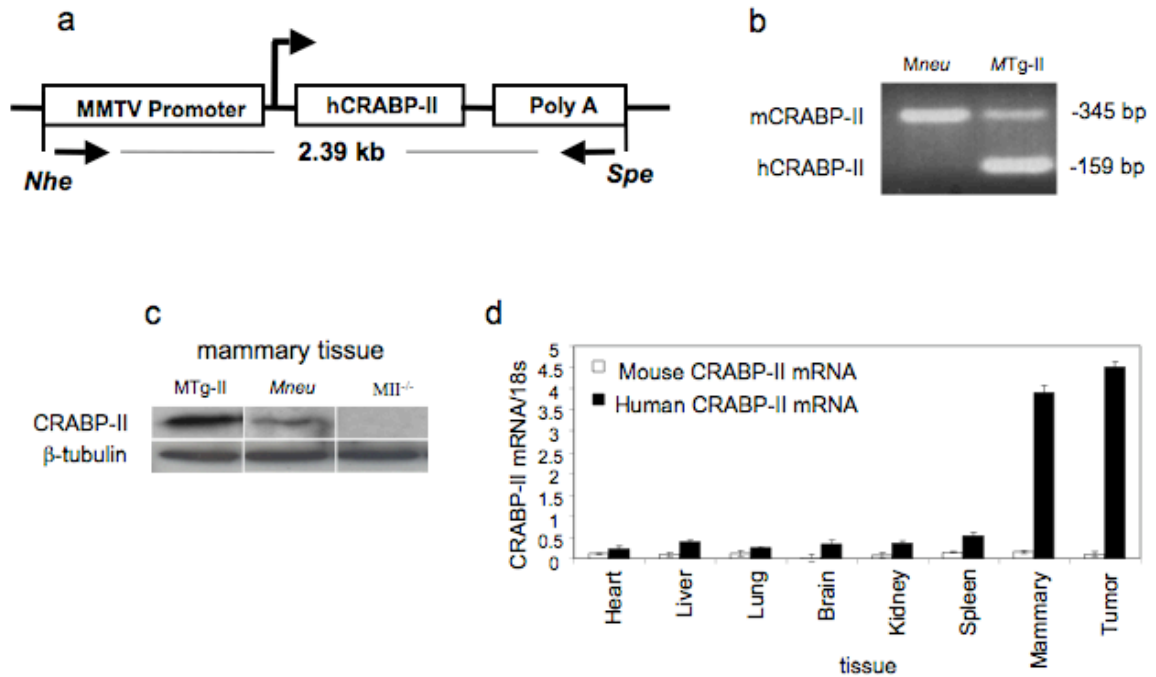


Figure 3.1. Generation of transgenic mice. (a) Mice over-expressing CRABP-II in the mammary gland (*MMTV-CRABP-II*) were generated using a targeting construct consisting of the mammary tissue-specific promoter/enhancer (MMTV-LTR) derived from the mouse mammary tumor virus, a synthetic human CRABP-II cDNA construct, and a mammalian beta globin polyadenylation signal. (b) Primer-specific detection of hCRABP-II transgenic construct. (c) Expression levels of CRABP-II in mammary tissue of *MMTV-neu* (*Mneu*) *MMTV-neu/MMTV-CRABP-II* (MTg-II), and *MMTV-neu-CRABP-II*^{−/−} (*MII*^{−/−}) mice were assessed by immunoblotting. (d) Expression levels of mRNA for hCRABP-II and mCRABP-II in transgenic and control mouse tissues, measured by Q-PCR and normalized to 18s mRNA. Data are mean±SEM, n=3.

Expression levels of RA receptors and binding proteins in tumors that develop in the mouse models.

To begin to characterize RA signalling in tumors that develop in *MMTV-neu* mice and in their counterparts that either over-express CRABP-II in mammary tissue, or lack the protein, the levels of expression of the two RA receptors, RAR and PPAR β/δ , and the two RA binding proteins, CRABP-II and FABP5, were examined (Fig. 3.2). As previously noted (Schug et al., 2007), carcinogenesis in *MMTV-neu* mice is accompanied by downregulation of CRABP-II and upregulation of FABP5 (Fig. 3.2a and 3.2d). As expected, the expression of CRABP-II was not detectable in tumors of the MCRABP-II^{-/-} mice, and was markedly elevated in mice that transgenically over-express the protein in mammary tissue (Fig. 3.2a).

Examination of the level of expression of RAR by immunoblots utilizing RAR antibodies that recognize all three RAR subtypes revealed that this receptor is down-regulated upon tumor development in *MMTV-neu* mice (Fig. 3.2b). Q-PCR analyses showed that expression of all three RAR isotypes are lower in tumors as compared to normal mammary tissue, and that RAR β displayed the largest response, showing a 40-50% lower level in tumors relative to normal mammary tissue (Fig. 3.2c).

Importantly, similar expression levels of RAR mRNAs were observed in all three mice models, indicating that they can be directly compared. RAR expression could not be restored by RA treatment of the mice (Fig. 3.2c). Expression levels of both FABP5 and PPAR β/δ were similar in all three mouse models (Fig. 3.2d-3.2f). Hence, tumors that develop in the three *MMTV-neu* mouse models express similar levels of RARs and PPAR β/δ , but they display large differences in the ratio of expression of CRABP-II and FABP5.

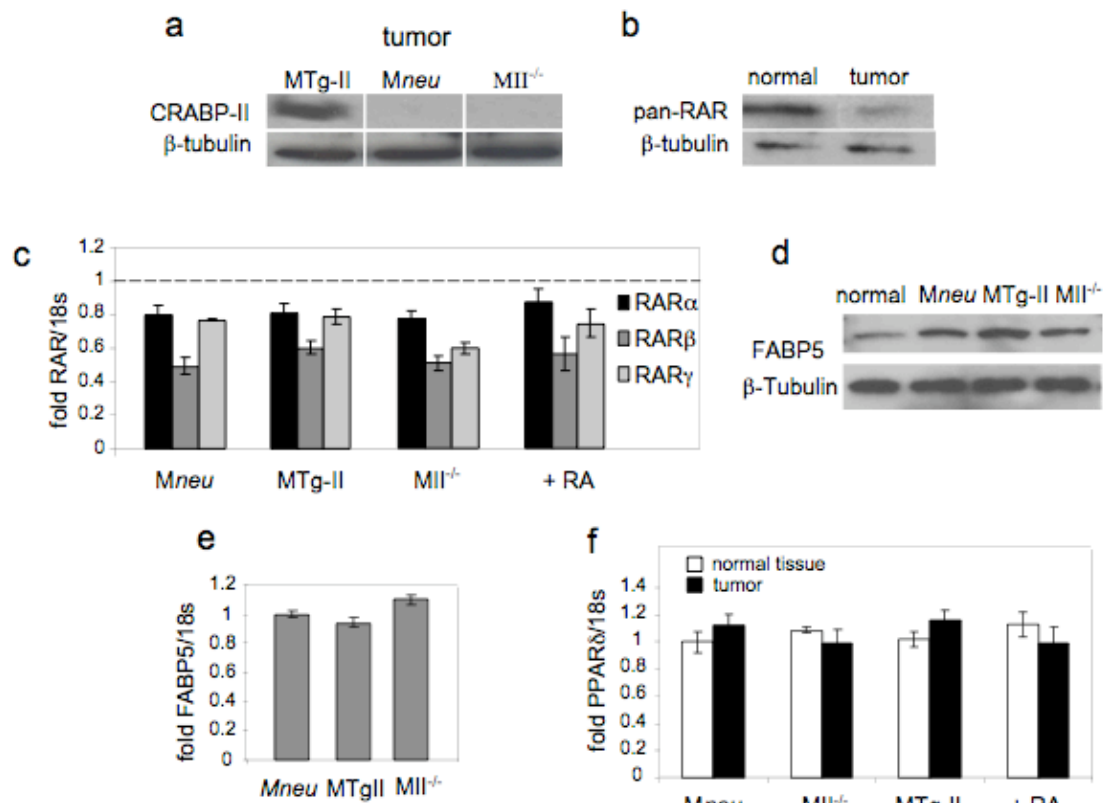


Figure 3.2. Levels of RA receptors and binding proteins in tumors that develop in the mouse models. (a.) Immunoblots of CRABP-II measured in tumors of *MMTV-neu* (Mneu), *MMTV-neu/MMTV-CRABP-II* (MTg-II), and *MMTV-neu/CRABP-II^{-/-}* (MII^{-/-}) mice. (b.) Expression levels of total RAR in normal mammary tissue and in mammary tumor in *MMTV-neu* mice, measured by immunoblotting. (c.) Expression levels of mRNA for RAR isotypes (α , β , γ) in tumors that arose in the denoted mouse models, and in *MMTV-neu* mice subjected to systemic RA as of age 140 d (+RA), measured by Q-PCR. (d) Immunoblots of FABP5 in normal mammary tissue, and in tumors that arose in the denoted mouse models. (e) Expression of FABP5 mRNA in tumors that arose in the denoted mouse models. (f) Expression levels of PPAR β/δ mRNA in tumors that arose in the mouse models and in *MMTV-neu* mice treated with RA. Levels of mRNA were normalized to 18s mRNA.

In *MMTV-neu* mice tumors, RA signals through PPAR β/δ , and is diverted to RAR upon increasing the CRABP-II/FABP5 ratio. To examine the possibility that the RA-resistance of tumors that arise in *MMTV-neu* mice may stem from RA signalling through PPAR β/δ , mice were systemically treated with RA (see Experimental Procedures), tumors were allowed to develop, and the intra-tumor expression levels of known target genes for RAR and PPAR β/δ were measured. Tumors with 1 cm³ volumes were removed, RNA extracted, and gene expression was assessed by Q-PCR. The data (Fig. 3.3a) showed a marked RA-induced upregulation of mRNA for the direct PPAR β/δ target genes fasting-induced adipose factor (*FIAF*, (Kersten et al., 2000)) and adipose differentiation-related protein (*ADRP*, (Schmuth et al., 2004)), as well as *PDK1*, a kinase that activates the Akt-1 survival pathway and thus mediates potent anti-apoptotic activities (Di-Poi et al., 2002; Tan et al., 2001). In contrast, RA treatment had little effect on the expression of the direct RAR target genes *caspase 9* and *BTG2*, or the indirect target *cyclin D1* (Donato and Noy, 2005; Donato et al., 2007) (Figure 3.3b). Hence, RA in these tumors does not activate RAR but, instead, induces transcriptional activation by PPAR β/δ .

To examine whether alteration in the expression ratio of CRABP-II and FABP5 modulates the ability of RA to activate the two receptors, expression levels of target genes for RAR and for PPAR β/δ were measured in tumors that arise in *MMTV-neu* mice that over-express CRABP-II and thus have a low FABP5/CRABP-II ratio (MTgCRABP-II), and in mice that lack CRABP-II (MCRABP-II^{-/-}). The data (Fig. 3.3c) demonstrated that the expression of all three PPAR β/δ target genes was significantly reduced in the presence of a low FABP5/CRABP-II ratio, and was upregulated in the absence of CRABP-II. In contrast, the direct RAR target genes *caspase 9* and *BTG2* were markedly upregulated upon over-expression of CRABP-II, and their expression decreased in mice lacking this binding protein (Fig. 3.3d). Another RAR-controlled gene involved in cell cycle regulation is *cyclin D1*, a down-

stream effector whose expression is down-regulated by BTG2 (Donato et al., 2007; Guardavaccaro et al., 2000; Kawakubo et al., 2004). Over-expression of CRABP-II decreased cyclin D1 expression, while ablation of this binding protein results in an increased level. Hence, a low FABP5/CRABP-II ratio depresses the expression of PPAR β/δ target genes including anti-apoptotic genes, and leads to gene expression commensurate with cell cycle arrest and enhanced apoptotic responses. Interestingly, Apaf1, the major protein in the apoptosome, is not subject to regulation by RAR, but it has been reported that its expression is upregulated in cultured mammary carcinoma cells ectopically overexpressing CRABP-II. It has been further shown that this effect is exerted in the absence of RA (Donato and Noy, 2005). The observations that Apaf1 expression is also markedly upregulated upon over-expression of CRABP-II *in vivo* (Fig. 3.3d) suggests that, in addition to its cooperation with RAR, this binding protein may contribute to apoptotic responses through another, as yet unknown, mechanism.

Changing the CRABP-II/FABP5 ratio alters RA activities in cell lines generated from tumors of *MMTV-neu* mice. The role of the CRABP-II/FABP5 ratio in regulating cellular responses to RA was further investigated by utilizing cell lines generated from tumors that developed in *MMTV-neu* mice with varying FABP5/CRABP-II ratios (see Experimental Procedures for details).

In accordance with the expression profile *in vivo*, NaF cells, generated from *MMTV-neu* tumors (Elson and Leder, 1995), display a high FABP5/CRABP-II ratio ((Schug et al., 2007) and Fig. 3.4a, 3.4b). Cells generated from *MMTV-neu* mice with transgenic mammary over-expression of CRABP-II and from MCRABP-II^{-/-} mice similarly retained the binding protein expression profiles observed *in vivo* (Fig. 3.4a, 3.4b). Also similarly to the *in vivo* behavior, CRABP-II-null cells displayed a decreased expression of the RAR target gene *Cyp26a* (White et al., 2000), and an increased expression of the PPAR β/δ target *PDK1* as compared with cells over-

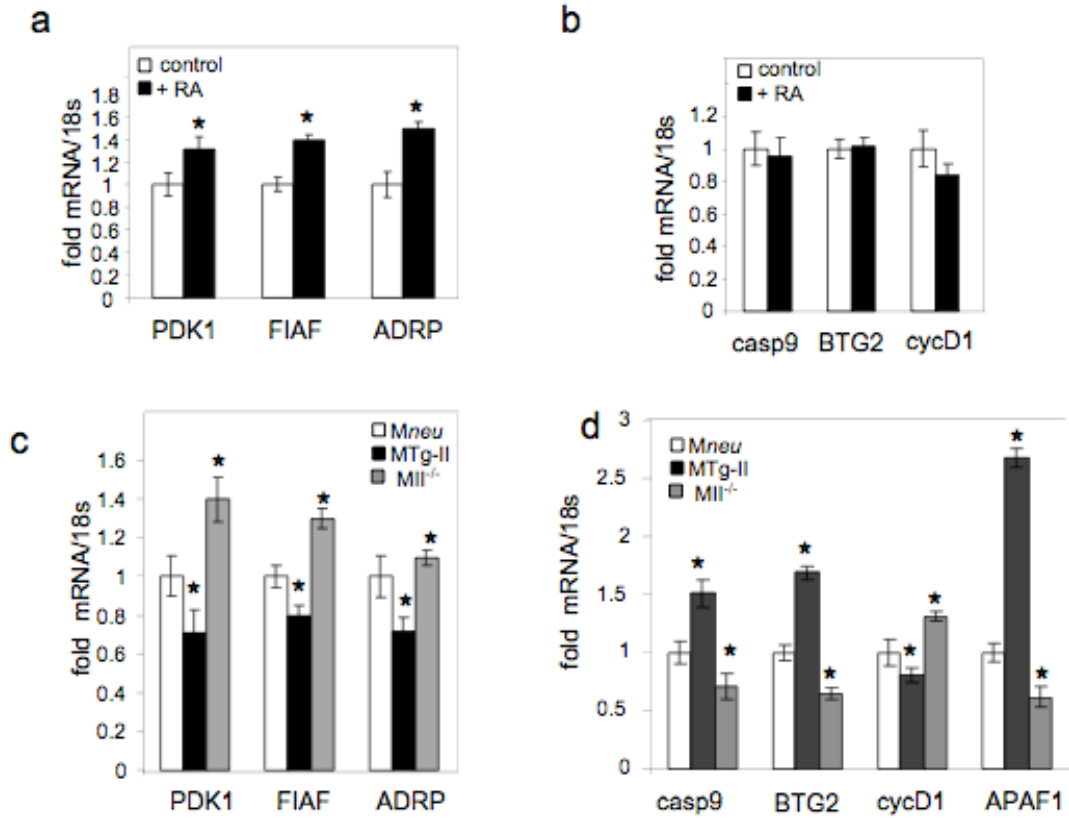


Figure 3.3. Increasing the CRABP-II/FABP5 ratio in *MMTV-neu* mice directs RA from PPAR β/δ to RAR. (a and b) Expression levels of mRNA for the denoted genes in tumors that arose in untreated female *MMTV-neu* mice and in mice systemically treated with RA as of age 140 days. (a.) Levels of mRNA for PPAR β/δ target genes. * $p < 0.01$ vs.untreated mice; (b.) Levels of mRNA for RAR-controlled genes. (c and d) Expression levels of mRNA for target genes for PPAR β/δ (c) and RAR (d) in tumors thsat arose in the denoted mouse models. * $p < 0.01$ as compared to *MMTV-neu* controls. Levels of mRNA were normalized to 18s mRNA. Data (mean \pm SEM) were analyzed with 2-tailed Student's t tests with pool variances (n=3).

expressing this binding protein (Fig. 3.4c). RA treatment upregulated the expression of the RAR target gene in cells with a high CRABP-II level, but induced the PPAR β/δ target in CRABP-II^{-/-} cells (Fig. 3.4c).

To examine the functional consequences of these differential transcriptional activities, the ability of RA to inhibit the growth of the cell lines was assessed by measuring incorporation of bromodeoxyuridine (BrdU), which reports on DNA synthesis and thus on cell proliferation. Cells that over-express CRABP-II displayed a lower proliferation index and were markedly more sensitive to RA-induced growth inhibition as compared with cells lacking this binding protein (Fig. 3.4d). The role of the binding proteins was further investigated by examining the ability of RA to induce apoptosis. Cells were treated with either RA or the apoptosis inducer TRAIL, and apoptosis was assessed by monitoring the cleavage/activation of caspase 3 (Fig. 3.4e and 3.4f). In cells expressing a high CRABP-II/FABP5 ratio, both TRAIL and RA induced apoptosis. Strikingly, cells lacking CRABP-II displayed a markedly lower level of cleaved caspase 3, and RA, unlike TRAIL, decreased this level even further. Hence, RA induces apoptosis in the presence of a high CRABP-II/FABP5 ratio, but becomes an anti-apoptotic agent in cells in which this ratio is reversed.

Decreasing the FABP5/CRABP-II ratio induces apoptosis and suppresses mammary tumor growth in MMTV-*neu* mice. These observations suggest that decreasing the FABP5/CRABP-II ratio diverts RA from PPAR β/δ to RAR, and that this ‘switch’ results in growth inhibitory activities. To examine this notion, the rate of growth of mammary tumors in MMTV-*neu* mice with varying levels of expression of CRABP-II was studied. Measurements of tumor size were initiated when tumors reached a volume of 0.065cm³. The data (Fig. 3.5a, 3.5b, and Table 3.1) showed that tumor growth in CRABP-II-null was significantly facilitated, while mammary over-

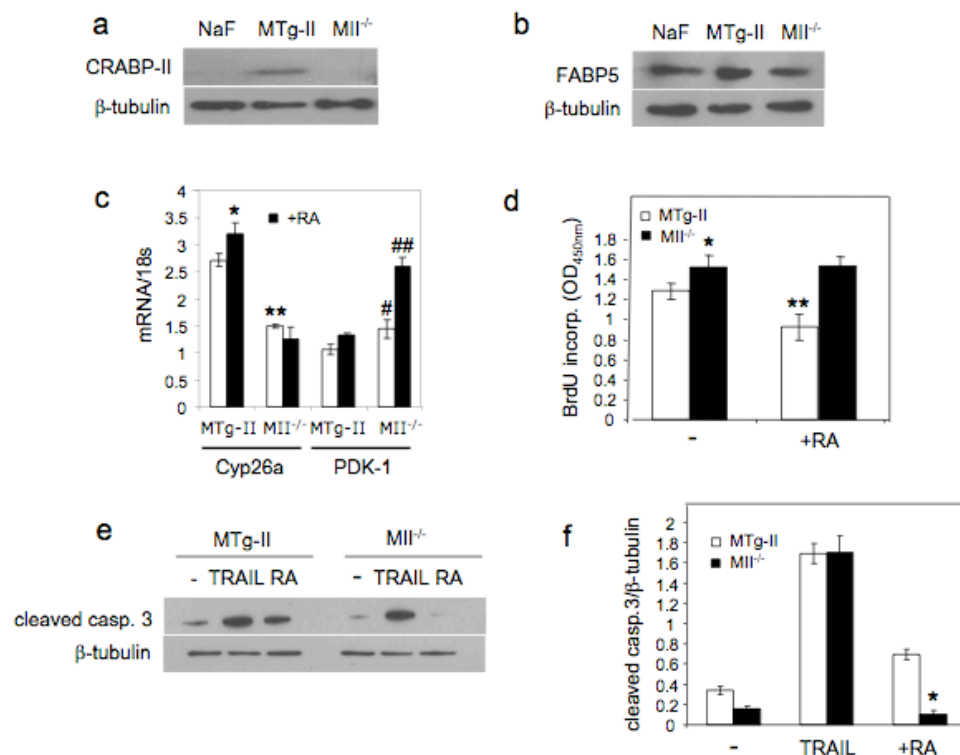


Figure 3.4. Effects of varying the CRABP-II/FABP5 ratio on RA activities in cell lines derived from *MMTV-neu* mice tumors. (a,b) Immunoblots of CRABP-II (a) and FABP5 (b) in cell lines derived from tumors that arise in *MMTV-neu* (NaF), *MMTV-neu/MMTV-CRABP-II* (MTg-II), and *MMTV-neu/CRABP-II^{-/-}* (MII^{-/-}) mice. (c) Expression levels of mRNA for the RAR target gene *Cyp26a*, and the PPARβ/δ target *PDK-1* in the denoted cell lines. Cells were treated with 0.1 μM RA for 4 hr., lysed, and total RNA extracted and analyzed by Q-PCR. *p=0.05 and **p<0.001 as compared to untreated MTg-II cells. #p<0.01 as compared to untreated MTg-II cells; ##p<0.01 as compared to untreated MII^{-/-} cells. (d) BrdU incorporation. Denoted cell lines were treated with 1 μM RA for 24 hr., cell proliferation was monitored using a BrdU cell proliferation assay (Calbiochem). *p=0.03 and **p=0.02 as compared to untreated MTg-II cells. (e) Denoted cell lines were with RA (1 μM, 12 hr.) or TRAIL (10 ng/ml, 12 hr). Cells were lysed and the level of cleaved caspase 3 assessed by immunoblots. (f) Quantitation of the experiment described in (e).

expression of CRABP-II markedly delayed tumor growth. Mice with an elevated mammary CRABP-II/FABP5 ratio also displayed a lower number of tumors per mice, and, strikingly, while tumors developed in 100% of mice in other groups, 4 out of 13 MTg-CRABP-II mice remained tumor-free throughout the duration of the experiment (Table 3.1).

Table 3. 1 Tumor growth in MMTV-neu mice with varying CRABP-II expression levels

genotype	mice with tumors	tumors/mice	tumor volume day 20 (cm ³ , mean±SE)*	median survival (days)**
<i>MMTV-neu</i>	22/22	2.3	0.68±0.20	399
MCRABP-II ^{-/-}	9/9	2.3	0.80±0.23	326
MTg-CRABP-II	9/13	1.3	0.32±0.09	513

Number and volumes of tumors arising in the different mouse models. *Tumor volumes are mean ±SE, p= 0.00256 comparing MTg-CRABP-II to MCRABP-II^{-/-}, and p=0.000321 comparing MTg-CRABP-II to *MMTV-neu* mice. **Survival rate of mice was defined as the age in which tumor reached 0.524 cm³. p=0.005 and =0.013 comparing MTg-CRABP-II and MCRABP^{-/-} to *MMTV-neu* controls, respectively. Survival fractions were calculated using the Kaplan-Meier method. Survival curves were compared by log rank Mantel-Haenszel tests. Means were compared by estimation of the two-tailed P with unpaired t tests.

All mammary neoplasms in the three cohorts of mice were adenocarcinomas, however, some differences were observed. In agreement with previous studies of the *MMTV-neu* model (Cardiff et al., 2000; Cardiff et al., 1991), adenocarcinomas in the control mice were composed of solid sheets of neoplastic epithelial cells with little or no glandular differentiation (Fig. 3.5c). Neoplastic population consisted of uniform densely packed relatively small cells with eosinophilic cytoplasm and rounded to elongated nuclei with diffuse chromatin pattern. Mitotic figures were observed often alongside with few apoptotic cells or bodies. In MTg-CRABP-II mice, the majority of tumors (16 out of 18) contained areas of adenocarcinomas with larger cells organized in nests or bundles separated by thin bands of connective tissue and blood vessels (Fig. 3.5d). The nuclei of the tumor cells were larger, with more open chromatin pattern, giving them vesicular appearance with prominent nucleoli and clumped heterochromatin. Such areas varied from 10-30% to over 90% of neoplasms. Single mitotic figures as well as apoptotic cells were seen. The adenocarcinomas in the MCRABP-II^{-/-} mice were with solid/nodular growth, more similar to *MMTV-neu* model. Their cells were densely packed, with pale eosinophilic cytoplasm and scanty connective tissue. In 8 out of 12 tumors there were areas of cells with nuclei of intermediate size and open chromatin pattern (Fig. 3.5e). Numerous mitoses and few apoptotic cells were seen.

To obtain further insight into the mechanism through which alteration in the FABP5/CRABP-II ratio modulate tumor growth, proliferation and apoptosis in tumors that arise in the different mouse models were evaluated. Immunostaining for the proliferation marker Ki67 (Fig. 3.6a-3.6c and 3.6g) showed a significant decrease in proliferation neoplasms of mice over-expressing CRABP-II, and a slight increase

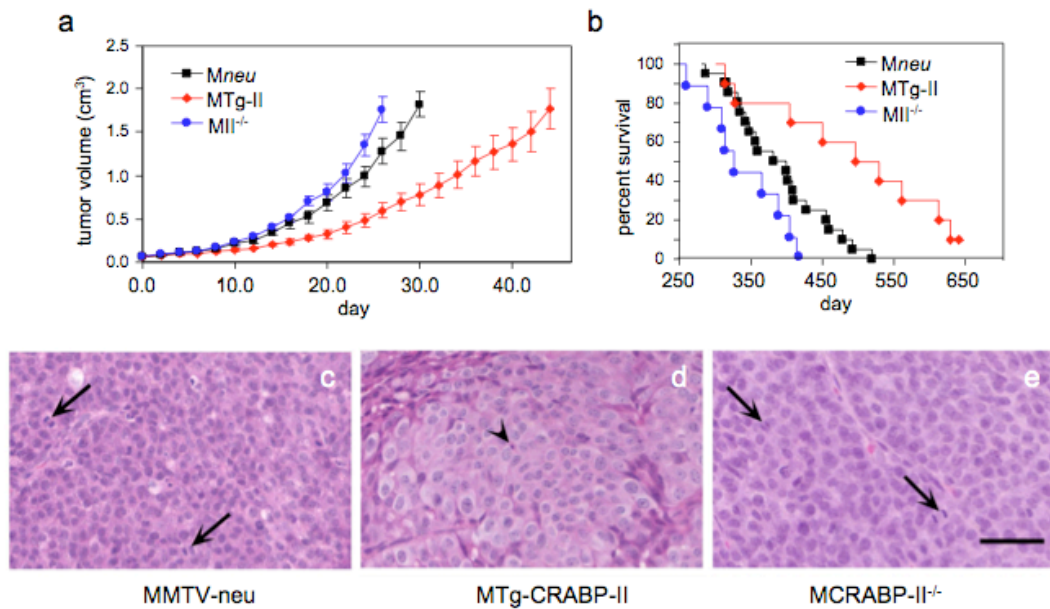


Figure 3.5. Mammary tumor growth in *MMTV-neu* mice expressing varying *FABP5/CRABP-II* ratios. Tumor growth was monitored in female *MMTV-neu*^{+/-} (*MMTV-neu*), *MMTV-neu/MMTV-CRABP-II* (MTg-CRABP-II), and *MMTV-neu/CRABP-II*^{-/-} (MCRABP-II^{-/-}) mice. (a) Tumor sizes in the denoted groups were measured with calipers with a start point (day 0) of tumor volume = 0.065 cm³. (b) Kaplan-Meier survival curves. Mouse survival was defined as the age in which tumor volume reached 0.524 cm³. (c-e) Hematoxylin and eosin staining of mammary tumors that arose in the mouse models. (c) Mammary carcinoma in an *MMTV-neu* mouse. Solid growth of dense relatively small tumor cells with round dark nuclei. Mitotic figures (arrows) and few apoptotic cells (arrowhead) are seen. (d) Mammary carcinoma in an MTg-CRABP-II mouse. The cells are larger, with vesicular nuclei and prominent nucleoli and heterochromatin. They are arranged in small nests or bundles separated by thin connective tissue bands. Apoptotic cells (arrowhead) are seen. (e) Mammary carcinoma in an MCRABP-II^{-/-} mouse. Solid growth of cells with dense chromatin and frequent mitotic figures (arrows). Bar, 75 μm.

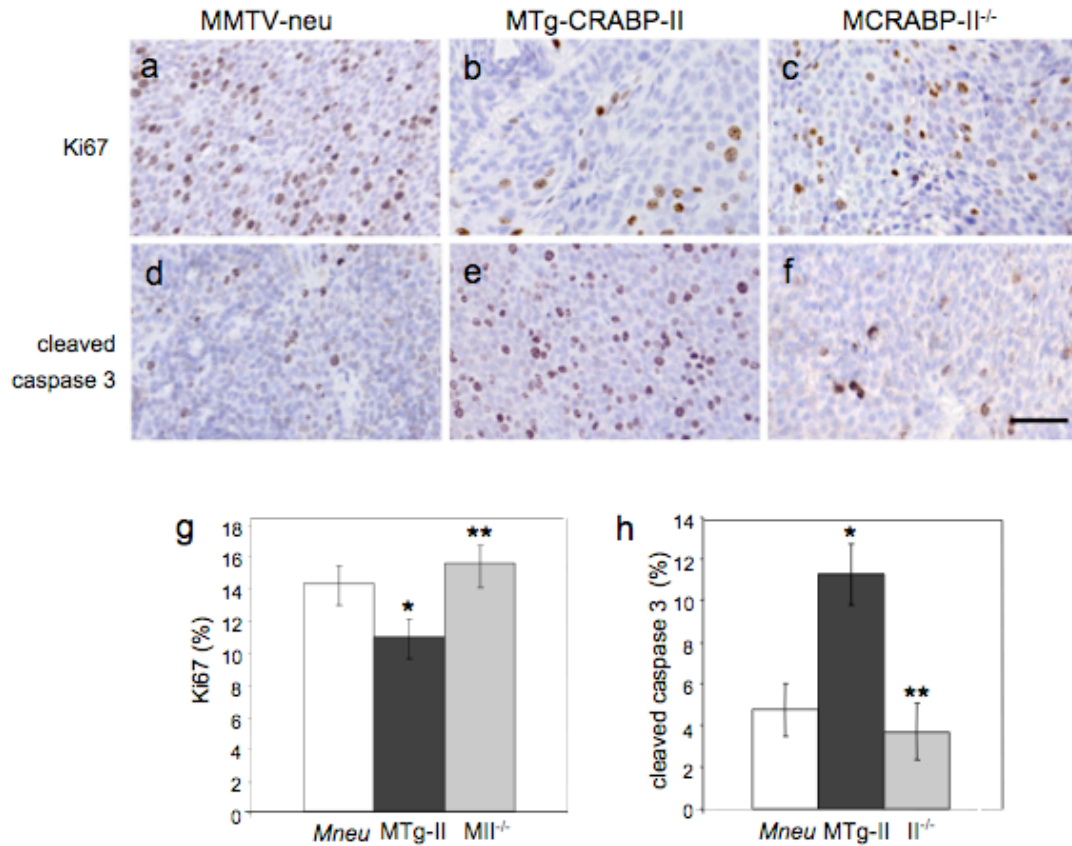


Figure 3.6. Varying the FABP5/CRABP-II ratio affects proliferation and apoptosis of neoplastic cells in tumors of *MMTV-neu* mice. (a-c.) Proliferation of tumor cells detected by immunostaining with antibodies toward Ki67 (brown). (a) An *MMTV-neu* tumor; (b) An *MTg-CRABP-II* tumor; (c). An *MCRABP-II^{-/-}* tumor. (d-f) Detection of apoptotic cells by immunostaining for cleaved-caspase 3 (brown). (d) An *MMTV-neu* tumor; (e) An *MTg-CRABP-II* tumor; (f) An *MCRABP-II^{-/-}* tumor. Calibration bar, 75 μm. (g) Quantitation of proliferation levels measured as the percentage of Ki67-stained cells. * $p=0.022$, ** $p=0.057$ vs. *MMTV-neu* controls. (h) Quantitation of apoptotic index measured as the percentage of cleaved caspase-3-stained cells. * $p=0.002$, ** $p=0.067$ vs. *MMTV-neu* controls. Quantitations were performed as described under Experimental procedures. Data (mean±SEM) were analyzed with 2-tailed Student's t tests with pool variances ($n=3$).

in proliferation in the CRABP^{-/-} tumor. On the other hand, the fraction of apoptotic cells, evaluated by immunostaining for cleaved caspase 3, was markedly higher in mammary tumors arising from mice overexpressing CRABP-II compared to either the CRABP-II-null mice or the MMTV-neu controls (Fig. 3.6d-3.6f, and 3.6h).

3.5 Discussion

RA displays pronounced anticarcinogenic activities and is currently used in therapy of some cancers and is being tested for treatment of others. Most notably, the ability of RA to target the oncogenic promyelocytic leukemia-RAR fusion protein renders RA a powerful therapeutic agent in treatment of PML (Lengfelder et al., 2005).

However, RA therapy of cancer is hampered both by pronounced toxicity and by the development of RA-resistance in tumors and, consequently, general use of this agent in oncology remains limited. RA-resistance in tumors has been shown to originate from various defects in RA signalling but remains incompletely understood.

Moreover, in some cases, RA treatment not only fails to inhibit carcinoma cell growth but, instead, results in facilitated tumor development, as exemplified by the marked RA-induced acceleration of mammary tumor development in the mouse model of breast cancer *MMTV-neu* (Schug et al., 2007).

It is well established that many cellular responses to RA are mediated by the nuclear receptor RAR, which, in various carcinomas, upregulates the expression of genes that evoke differentiation, cell cycle arrest, and apoptosis, thereby inhibiting cell growth. The ability of RA to function in a contrary fashion, i.e. to enhance tumor development, is thus unlikely to be mediated by RAR, and the basis for this behavior has long remained an enigma. The recent reports that, in addition to activating RAR, RA also serves as a ligand for PPAR β/δ (Schug et al., 2007; Shaw et al., 2003), a nuclear receptor that targets genes involved in mitogenic responses and in survival pathways (Di-Poi et al., 2002; Icre et al., 2006; Tan et al., 2004; Wang et al., 2006),

suggests a possible mechanism through which RA may facilitate tumor growth. Importantly, it was also suggested that the partitioning of RA between the two receptors and thus, regulation of anti-proliferative vs. proliferative responses to RA, is controlled not by the receptors themselves but by two intracellular lipid-binding proteins that deliver the ligand to them: CRABP-II which channels RA to RAR, and FABP5 which specifically shuttles RA to PPAR β/δ (Schug et al., 2007). We thus set out to explore the hypothesis that the facilitated development of mammary tumors in *MMTV-neu* mice in response to RA may originate from an aberrantly high FABP5/CRABP-II expression ratio in these tumors which, in turn, may channel RA to PPAR β/δ rather than to RAR.

The data show that this is indeed the case. Examination of gene expression profiles in tumors that arise in female *MMTV-neu* mice revealed that, in these tumors, RA upregulates the expression of PPAR β/δ target genes, including the survival factor *PDK1*, but is not able to induce the expression of cell cycle control and apoptotic RAR targets. Increasing the already high FABP5/CRABP-II ratio results in further elevation of PPAR β/δ targets and further decrease in genes regulated by RAR. On the other hand, a decreased FABP5/CRABP-II ratio in tumors diverted RA from PPAR β/δ to RAR, upregulated the apoptosis factor caspase 9 and the cell cycle control protein BTG2, and downregulated cyclin D1. Correspondingly, neoplastic cells in mammary carcinomas of *CRABP-II*^{-/-} and *MMTV-neu* mice displayed numerous mitotic figures and a high proliferation rate, while those of tumors that overexpress CRABP-II were characterized by reduced proliferation and a high apoptotic rate. Interestingly, slower growth of neoplasms that overexpress CRABP-II was accompanied by changes in morphology and growth pattern of neoplastic cells, which may indicate a more differentiated state. Lowering the FABP5/CRABP-II ratio thus attenuated proliferation, enhanced apoptosis, changed cell morphology and markedly inhibited tumor growth.

Varying the expression level of CRABP-II in mammary tumors also markedly affected the expression Apaf1, the major protein in the apoptosome. The effect recapitulates *in vivo* the previously reported observation that CRABP-II expression upregulates Apaf1 in MCF-7 mammary carcinoma cells. In the cultured cells, this effect was found to take place in the absence of RA and it was thus suggested that the activity stems from an RAR-independent activity of the binding protein (Donato and Noy, 2005). Hence, pro-apoptotic activities of CRABP-II may originate both from its cooperation with RAR and from an additional function, the nature of which is currently unknown.

Remarkably, the FABP5/PPAR β/δ pathway was found to facilitate, and CRABP-II/RAR signalling suppressed tumor growth in the absence of ectopic administration of RA, i.e. at the level of RA endogenously present in the mouse. Hence, strategies that target the two binding proteins may dramatically reduce the doses of RA needed for efficacious treatment, and bypass both the RA-resistance of tumors and the toxicity barrier encountered in current therapies. These observations point at the conclusion that CRABP-II functions as a potent tumor suppressor, and suggest further that inhibition of FABP5 may comprise a novel strategy for treatment of some cancers. While no antagonists for this protein currently exist, a recent report demonstrated the development of a small molecule inhibitor for the homologous protein FABP4 (A-FABP, aP2). In the case of FABP4, it was shown that such an inhibitor is an effective therapeutic agent against atherosclerosis and type 2 diabetes in mouse models (Furuhashi et al., 2007). Together with the data reported here, these observations suggest that compounds that target intracellular lipid-binding proteins may comprise a new class of therapeutic agents. Development of such compounds could be greatly assisted by the recent delineation of the structural features that underlie the ability of specific ligands to activate intracellular lipid-binding proteins, including CRABP-II, FABP5 and FABP4 (Ayers et al., 2007; Gillilan et al., 2007;

Sessler and Noy, 2005).

3.6 Experimental Procedures

Reagents. Antibodies against CRABP-II were provided by Cecile Rochette-Egly (Gaub et al., 1998). Antibodies against FABP5, RAR, and cleaved caspase 3 were purchased from R&D, Santa Cruz Biotechnology, and Cell Signaling Technologies, respectively. Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase-conjugated antibody were purchased from Amersham/GE Healthcare Life Sciences and BioRad Laboratories, respectively. RA and TRAIL were from Calbiochem and Sigma Chemical Co., respectively. RA was dissolved in ethanol.

Construction of the MMTV-CRABP-II transgene and generation of transgenic mice. To construct the MMTV-CRABP-II-beta globin pA transgene, the 414 bp hCRABP-II EcoR1-BamH1 fragment from pSG5 was ligated to the 427 bp BgIII-Pac1 human beta globin polyadenylation signal fragment from pBroad, and cloned into pMAMneo containing the mammary epithelium specific promoter/enhancer MMTV-LTR. The 2.39-kb MMTV-LTR-CRABP-II beta globin pA transgene was released by restriction with Nhe and Spe, purified, and injected into the pronuclei of fertilized FVB/N oocytes by the Transgenic Facility of Cornell University College of Veterinary Medicine. Transgenic founders were identified by PCR, and copy number was confirmed by Real Time quantitative PCR (Q-PCR).

Mouse breeding . Colonies of MMTV-*neu* and MMTV-CRABP-II mice were maintained on FVB/N background. CRABP-II^{-/-} mice were on a C57BL/6 background.. The MMTV-*neu*^{+/-}/CRABP-II^{-/-} model was established by crossing FVB/N-Tg(MMTV*neu*)202Mul/J (MMTV-*neu*; Jackson Laboratories, stock #002376) mice, which are homozygous for the *neu* transgene (*neu*^{+/+}), with C57BL/6 CRABP-II^{-/-} mice (provided by the laboratory of Pierre Chambon (Lampron et al., 1995)). The first generation of crossed mice (F1) were bred with CRABP-II^{-/-} males to yield

the MMTV-*neu*^{+/-}/CRABP-II^{-/-} cohort (termed MCRABP^{-/-} in this manuscript), and MMTV-*neu*^{+/-}/CRABP-II^{+/-} littermates, which were used as a control (termed MMTV-*neu*). Initial studies demonstrated that rate of mammary carcinogenesis on the F1 FVB/NXC57BL/6 background is similar to that on FVB/N background, and, thus, is appropriate for described experiments (not shown). MMTV-CRABP-II mice were crossed with MMTV-*neu* mice to generate MMTV-*neu*^{+/-}/MMTV-CRABP-II animals (termed here MTgCRABP). All mice were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee.

Genotyping. DNA was isolated essentially as described (Nikitin et al., 1993).

Transgenic mice were identified by PCR using primers for CRABP-II cDNA were 5'-AGC AGT GGA GAT CAA ACA GGA GGG A-3' (CRABP-II hex2-5', sense), 5'-CAG CAG TCG AGA TCA AAC AGG AGA A-3' (CRABP-II mex2-5', sense), and 5'-AAG TTC CCA CTC TCC CAT TTC ACC A-3' (CRABP-II uex3-3', antisense).

Primers CRABP-II hex2-5' and CRABP-II mex2-5' are specific for human and mouse exon 2, respectively. The primer CRABP-II uex3-3' recognizes both human and mouse sequence in exon 3 (Manor et al., 2003). Amplification of genomic and cDNA results in 345-bp and 159-bp PCR fragment, respectively. MMTV-*neu*/CRABP-II^{-/-} transgenic mice were identified by primers CRABP-II5' (5'-TAC TGA TGC GGG AGA AGG GTA TCT T-3'), CRABP-II3' (5'-GCC AGA AAA GTT AGG CA TAGT GGC A-3'), TKP13' (5'-GCC TGC TCT TTA CTG AAG GCT CTT T-3'). The MMTV-*neu* transgene was identified by primers Neu5' (5'-GCC AGC CCG GTG ACA TTC ATC ATT G-3'), and Neu 3' (5'-CCG CAT CTG AGC CTG GTT GGG CAT T-3') (Nikitin et al., 1993). The PCR temperature profile was 94°C for 30 s, 60°C for 1 min., and 72°C for 2 min. with extension of the last cycle for 10 min. at 72°C.

Biochemical procedures. Cell and tissue extractions and immunoblot analyses were performed as previously described (Schug et al., 2007). For real time quantitative

PCR (Q-PCR) analyses, RNA was extracted using RNeasy (Qiagen, Valencia, CA), and cDNA was generated using Gene Amp RNA PCR (Applied Biosystems, Foster City, CA). Q-PCR was carried out using TaqMan chemistry and Assays on Demand probes (Applied Biosystems) for CRABP-II ([Hs00275636_m1](#), Mm), PDK-1 (Mm00176884_m1), FIAF (Mm00480431_m1), ADRP ([Mm00475794_m1](#)), Cyp26a (Mm00514486_m1), BTG2 (Mm00476162_m1), cyclin D1 (Mm00432359_m1), RAR α (Mm00436264_m1), RAR β (Mm01319674_m1), RAR γ (Mm00441083_m1), PPAR β/δ (Mm00803186_g1), FABP5 (Mm00783731_s1). 18s rRNA (4319413E-0312010) was used as a control. Analyses were carried out using the relative standard curve method (Applied Biosystems Technical Bulletin no. 2).

Carcinogenesis studies. All experiments were performed on multiparous females bred thrice. RA was administered by 90-day release 15 mg pellets (Innovative Research of America, Sarasota, Florida) implanted s.c. Mammary tumor development was monitored by palpation three times per week, and tumor sizes were measured with calipers and recorded without investigator's knowledge of study groups.

Pathological assessment. Complete necropsies and gross and microscopic examination of mice and tissues were performed. Tissue samples from internal organs (heart, lung, liver, spleen, brain kidney, adrenal glands), mammary glands and tumors were collected, fixed in buffered paraformaldehyde overnight at 4°C and embedded in paraffin. Sections (4 μ m thick) were mounted on glass slides and stained with hematoxylin and eosin. For biochemical analyses a part of material was snap-frozen in liquid nitrogen.

Immunohistochemical analyses. Immunostaining of paraffin sections of paraformaldehyde-fixed tissue was performed by a modified avidin-biotin-peroxidase (ABC) technique (Nikitin et al., 2002). Antigen retrieval was done by boiling the

slides in 10 mM citric buffer (pH 6.0) for 15 min. Cells undergoing apoptosis were detected by immunohistochemical analyses using rabbit polyclonal antibody recognizing activated cysteinyl aspartic acid-protease-3 (cleaved caspase-3; Cell Signaling, #9661; dilution 1:200). Cell proliferation index was determined by Ki67 antibody staining (Novocastra Laboratories, #NCL-Ki67p; 1:1,000 dilution). The primary antibodies were incubated with deparaffinized sections for 1 hr. at room temperature. Sections were subsequently incubated for 30 min. at room temperature with biotinylated secondary antibody and subsequently detected with the ABC Elite kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine (Sigma, St. Louis, MO) as substrate.

Analyses of apoptosis and proliferation in tumors. Apoptotic and proliferation indices were determined essentially as described previously (Zhou et al., 2006). Briefly, images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using 40x objective under a Zeiss Axioskop 2 Plus microscope. In all experiments 10 images with area $10,384 \mu\text{m}^2$ were captured for each slide and transferred to Photoshop 6.0. 2,000 cells were counted in each experiment after overlaying a grid.

Cell lines. Cell lines with varying FABP5/CRABP-II ratios were generated from mammary tumors of *MMTV-neu*, *MCRABP-II^{-/-}* and *MTgCRABP-II* mice. Tumors were collected from all mouse cohorts after reaching a volume of 0.524 cm^3 , and cell lines were generated as previously described (Elson and Leder, 1995). Briefly, tumors were removed, rinsed several times in PBS, and placed in a dish containing 0.05% trypsin and 0.02% EDTA. Tumors were finely minced with a scalpel, pipetted up-and-down 6-7 times, and placed in an incubator for 15 min. at 37°C. The process was repeated twice. The contents of the dish was placed in a 50 ml tube, allowed to settle, and the supernatant removed. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin

was added to a volume of 25 ml. Mixture was centrifuged and the pellet resuspended in fresh media, transferred to a flask, and cells were grown undisturbed for 24 hr. Cells were passaged 25 times prior to experimentation. Cells were grown in DMEM supplemented with 10% charcoal-treated newborn bovine calf serum (Cocalico Biologicals Inc., Reamstown, Pa.), 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

BrdU cell proliferation assay. Cells were seeded in 96 well plates in DMEM supplemented with 2% charcoal-treated FBS and grown overnight. Cells were treated with 1 μ M RA for 24 hr. and analyzed using the BrdU cell proliferation assay (Calbiochem).

Statistical analyses. Statistical analyses was carried out using Prism 3.02 (Graphpad, Inc., San Diego, CA) software. Survival fractions were calculated using the Kaplan-Meier method. Survival curves were compared by log rank Mantel-Haenszel tests. Means were compared by estimation of the two-tailed P with unpaired t tests.

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CHAPTER FOUR

GENERAL DISCUSSION

Studies described in this dissertation aimed to delineate the molecular basis for the ability of retinoic acid (RA) to evoke opposing activities in different cells. This work focused on understanding the roles of the two RA nuclear receptors, RAR and PPAR β/δ , and the two RA-binding proteins, CRABP-II and FABP5, in regulating cellular responses to their ligand.

It is well established that RA, the active metabolite of vitamin A, influences biological processes by activating the retinoic acid receptors (RARs) (Chambon 1996; Donato and Noy 2005). Transcriptional activation of RAR has been shown to trigger differentiation, cell cycle arrest, and apoptosis. Accordingly, RA displays pronounced anticarcinogenic activities and is used or is being tested as a chemotherapeutic agent in several forms of human cancers (Soprano, Qin et al. 2004). Previous work from this laboratory revealed that CRABP-II, an intracellular lipid binding protein, functions to deliver RA to RAR in the nucleus, thus enhancing the receptor's transcriptional ability. Consequently, CRABP-II sensitizes carcinoma cells to the growth inhibitory activity of RA, an effect that was found mainly from induction of apoptosis (Budhu and Noy 2002; Manor, Shmidt et al. 2003; Donato and Noy 2005).

However, other observations demonstrated that, under some circumstances, RA activates pro-proliferate activities, and that these activities appear to be mediated through an RAR-independent pathway. A clue to the basis of this paradoxical activity stemmed from our previous observations that RA can activate a different nuclear receptor, namely PPAR β/δ . It was thus shown that RA binds to PPAR β/δ with a K_d of 15 nM, about an order of magnitude higher affinity than that displayed by PPAR α and PPAR γ (Shaw, Elholm et al. 2003). Accordingly, in the context of a reporter

construct, RA selectively activates PPAR β/δ but not other PPAR isotypes. Hence, this data suggests the possibility that, in some cells, RA may activate transcription not only through RAR but also through PPAR β/δ .

The observations that RA can activate both RAR and PPAR β/δ raised the questions of the factors that regulate the dual activity of this hormone. Previous studies have shown that RA is delivered to RAR by the intracellular lipid-binding protein CRABP-II. We show here, that, in a manner similar to that found for the cooperation of CRABP-II with RAR, this hormone is delivered to PPAR β/δ by another intracellular lipid-binding protein, namely, FABP5. Hence, the partitioning of RA between its two receptors is controlled by its two binding proteins. This behavior was found to lead to remarkable biological consequences. For example, we showed that, in keratinocytes, which express a high level of FABP5, RA activates PPAR β/δ and that the activation results in upregulation of the survival factor PDK1.

Consequently, RA protects these cells from apoptosis. Conversely, in MCF-7 mammary carcinoma cells, which express a high level of CRABP-II, RA activates RAR and thus triggers apoptosis. Moreover, we showed that modulating the relative expression of the two binding proteins profoundly changes the biological activity of RA. Hence, increasing the CRABP-II/FABP5 ratio in keratinocytes inverts the response of these cells and converts RA from a survival factor to a pro-apoptotic agent. Correspondingly, decreasing the ratio in MCF-7 cells converts RA from an apoptotic to an anti-apoptotic signal in these cells.

To examine whether CRABP-II and FABP5 control biological responses to RA *in vivo*, we used the mammary carcinoma mouse model MMTV*neu*. Assessment of the expression levels of RA-receptors in MMTV-*neu* tumors showed that PPAR β/δ levels remain unchanged and RAR is somewhat down-regulated upon tumorigenesis. Strikingly, tumor development was accompanied by marked down regulation of CRABP-II and an elevated level of FABP5. We hypothesized that RA-induced

proliferative responses in this tumors originated from the dysregulation of the binding proteins which, in turn, directs RA to PPAR β/δ . To investigate this hypothesis, we generated MMTV-*neu* mouse models with varied expression ratio of the two binding proteins. Examination of tumors that arise in these models revealed that the expression of three PPAR β/δ target genes was significantly reduced in the presence of a low FABP5/CRABP-II ratio, and was upregulated in the absence of CRABP-II. In contrast, the direct RAR target genes caspase 9 and BTG2 were markedly upregulated upon over-expression of CRABP-II, and their expression decreased in mice lacking this binding protein. Accordingly, measurements of tumors in these mouse models showed that tumor growth in the CRABP-II-null mouse model was significantly facilitated, while mammary over-expression of CRABP-II markedly delayed tumor growth.

In agreement with previous studies of CRABP-II over-expression in the MMTV-*neu* mouse model (Manor, Shmidt et al. 2003), we demonstrated a significant decrease in proliferation in mice over-expressing CRABP-II, and a slight increase in proliferation in the CRABP^{-/-} tumor. This behavior could be traced to a markedly higher apoptotic index in mammary tumors arising from mice overexpressing CRABP-II compared to either the CRABP-II-null mice or the MMTV-*neu* controls. The role of altering the CRABP-II/FABP5 ratio in regulating cellular responses to RA was further demonstrated by utilizing cell lines generated from tumors that developed in MMTV-*neu* mice with varying FABP5/CRABP-II ratios. The data showed that cells generated from MMTV-*neu* mice with transgenic mammary over-expression of CRABP-II and from CRABP-II^{-/-} mice retained the binding protein expression profiles observed *in vivo*. Also similarly to the *in vivo* behavior, CRABP-II-null cells displayed a decreased expression of the RAR target gene Cyp26a, and an increased expression of the PPAR β/δ target PDK1 as compared with cells over-expressing this

binding protein. Furthermore, RA treatment upregulated the expression of the RAR target gene in cells with a high CRABP-II level, but induced the PPAR β/δ target in CRABP-II^{-/-} cells. These results are the first *in vivo* demonstration of the importance of the CRABP-II/FABP5 ratio in regulating the transcriptional activity of RAR and PPAR β/δ , and thus the biological responses to their common ligand, RA.

The findings that a high FABP5/CRABP-II ratio directs RA to PPAR β/δ , and that, under such circumstances, RA protects cells against apoptosis and enhances proliferation suggest that while CRABP-II functions as a tumor suppressor, FABP5 may be an oncogene. Future studies will aim to determine whether this is the case and whether ablation of FABP5 or chemical inhibition of this protein may suppress tumor growth. In addition, it would be of great interest to explore whether dysregulation of expression of these iLBPs accompanies tumorigenesis in cancers other than the mammary cancer models used in the present work. Hence, strategies that target the two binding proteins may dramatically reduce the doses of RA needed for efficacious treatment, and bypass both the RA-resistance of tumors and the toxicity barrier encountered in current therapies.

Importantly, PPAR β/δ , in addition to its proliferative roles explored here, also participates in various metabolic functions. Of special interest in regard to this is the recent reports that activation of this receptor is protective against insulin resistance and adiposity, activities that are mediated by functions of the receptor in muscle and adipose tissue (Wang et al. 2003; Wang, Zhang et al. 2004; Lee, Olson et al. 2006). While we find here that RA can function as an endogenous ligand for this receptor, questions remain as to whether this hormone participates in regulating the metabolic functions of PPAR β/δ in muscle and adipose tissue. Finally, a key insight provided by our work concerns the importance of directed ligand transport in nuclear receptor activation. Future studies of this issue will aid in the understanding the pleiotropic effects of members of the nuclear receptor superfamily and their various ligands.

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